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Review

Modulation of protein biophysical properties by chemical glycosylation: biochemical insights and biomedical implications

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Abstract. Glycosylation constitutes one of the most important posttranslational modifications employed by biological systems to modulate protein biophysical properties. Due to the direct biochemical and biomedical implications of achieving control over protein stability and function by chemical means, there has been great interest in recent years towards the development of chemical strategies for protein glycosylation. Since current knowledge about glycoprotein biophysics has been mainly derived from the study of naturally glycosylated proteins, chemical glycosylation provides novel insights into its mechanistic understanding by affording control over glycosylation parameters. This review presents a survey of the effects that natural and chemical glycosylation have on the fundamental biophysical properties of proteins (structure, dynamics, stability, and function). This is complemented by a mechanistic discussion of how glycans achieve such effects and discussion of the implications of employing chemical glycosylation as a tool to exert control over protein biophysical properties within biochemical and biomedical applications.

Keywords. Biophysics, enzyme catalysis, glycoprotein, glycosylation, protease, protein dynamics, protein stability, protein therapeutics.

Introduction

Through the use of posttranslational modifications (e.g., phosphorylation, methylation), biological systems have managed to naturally evolve effective chemical strategies for the modulation of protein biophysical properties [1, 2]. Of these, glycosylation is one of the most common (>50% of all proteins are estimated to be glycosylated [3]) and important ones, as it serves to modulate a variety of biochemical processes, both at the cellular and protein level (Table 1) [4 – 16]. Accordingly, there is great interest in understanding the fundamental aspects of glycoprotein biophysics [17] due to the possible biochemical and biomedical implications involved in tailoring protein properties through engineered glycosylation [18]. *In vivo*, natural protein glycosylation occurs through enzymatic processes regulated by both glycosyltransferases and glycosidases [19]. This leads to the biosynthesis of highly complex mixtures of heterogeneously glycosylated proteins whose isoforms can contain variations not only in the amount, size, and charge state of the attached glycans but also in their regio- and stereo-chemistry [20]. Since control over the resulting glycan structural and spatial arrangement is believed to be one of the biggest challenges to the study and biomedical employment of glycopro-

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Table 1. Summary of biological processes modulated by glycosylation.

Cellular level	References	Protein level	References
Cell migration	[5, 6, 8, 14, 15]	Folding	[4, 13, 61–70]
Cell differentiation	[5, 8, 16]	Solubility	[4, 13, 178, 179]
Cell-cell signaling	[5, 6, 8, 10, 14–16]	Structural dynamics	[6, 20, 30, 41, 45–47, 52, 57]
Inflammatory response	[4, 5, 8]	Catalytic activity	[4–6, 30, 47, 49, 53, 57, 73–76]
Intracellular targeting	[4, 5, 8, 10]	Thermal stability	[4, 5, 13, 30, 47, 50–52, 55, 56, 60–62, 71, 72]
Blood-group antigenicity	[4, 6, 8, 10]	Kinetic stability	[56, 59, 171, 174, 175]
Immune regulation	[5, 8, 11]	Aggregation	[4, 13, 56, 158, 160, 176, 177]

teins [18], a multitude of alternative synthetic methods (e.g., chemoenzymatic and chemical) have been developed in recent years for the production of chemically glycosylated proteins [21–30]. However, most of the scientific insights concerning the effects of glycans on protein biophysics have been derived from the study of natural glycoproteins [31]. Although these studies have provided key insights on which protein properties can be modulated by glycosylation, further mechanistic understanding of the fundamental aspects of glycoprotein biophysics demands a detailed study of the influence that the glycosylation parameters (e.g., degree of glycosylation, glycan size) have on the properties of glycoproteins. This can be achieved through the use of chemical glycosylation [30, 32]. In this review, we present a survey of the effects of natural and chemical glycosylation on biophysical properties of proteins with emphasis on the changes caused by variations in glycosylation parameters. Additionally, we present some recent examples of how chemical glycosylation can be employed as a tool to control protein biophysical properties within biochemical and biomedical applications. In closing, we discuss how other biochemically and biomedically relevant chemical modifications (e.g., PEGylation) can affect protein biophysical properties.

Modulation of protein biophysical properties by glycosylation

Influence of glycosylation on protein structure and structural dynamics

The first observations of the presence of carbohydrate molecules in protein preparations can be traced as far back as 1888 to the work of Hammarsten on mucins [33] and 1929 to the work of Rimington on serum globulins [34]. However, the definitive nature of glycosylated proteins was not conclusively confirmed until the pioneering work of Albert Neuberger on ovalbumin (1938) [35, 36]. While subsequent studies attempted to structurally study glycoproteins by both chemical and spectroscopic methods [37–39], it was not until a series of studies performed on the submaxillary mucins by the Gerken research group (1986–1989) that the first detailed biophysical reports on the solution structural behavior of glycoproteins became available [40–43]. The primary purpose of these studies was to derive information on the effects of natural O-linked glycosylation on the structure and conformational dynamics of glycoproteins by comparing the nuclear magnetic resonance (NMR) relaxation behavior of native and sequentially deglycosylated submaxillary mucins [43]. From these studies it was concluded that glycosylation could decrease the structural mobility of the protein not only at the glycosylated residues but also at residues remote from the glycosylation sites without altering the overall structure [43]; a surprising conclusion due to the structurally complex nature of these proteins [mucins are composed of large molecular weight multimers (Mw $10^{5-}10^{7}$)] [44].

These initial results were later complemented by those of Dwek and collaborators using ribonuclease (RNase) [45–47]. A series of comparative NMR-derived structural studies between non-glycosylated RNase A and its naturally glycosylated isoform RNase B were performed based on the preceding studies performed by Puett on the same system [38]. RNase B is composed of a mixture of glycoforms in which one glycan chain is attached to the side-chain nitrogen of Asn_{34} with a variable glycan structural composition of $Man_nGlcNAc_2$ where n varies from 5 to 9 (Man: mannose; GlcNAc: N-acetylglucosamine) [48]. These structural studies were complemented by structural dynamic information of the different RNase isoforms derived from ^{1}H-NMR hydrogen-deuterium (H/D) exchange rates. The results led the researchers to arrive at the following conclusions about natural glycosylation on RNase structure: a) the presence of the glycan does not alter the overall structural fold of the protein, b) the glycan chain samples a substantial conformational space potentially shielding large por-

tions of the protein surface, and c) overall protein structural dynamics were slightly reduced by glycosylation since amide H/D exchange rates were slower in many regions of the protein including some more than 30 Å away from the Asn_{34} glycosylation site.

Further comparative studies were subsequently performed using other naturally glycosylated protein systems, such as plasminogen and tissue plasminogen activator [49], the adhesion domain of human CD2 [50], the free alpha subunit of human chorionic gonadotropin [51], and the proteinase inhibitor PMP-C (Pars intercerebralis major peptide C) [52]. In all of these studies a small reduction in protein structural dynamics was observed independent of the linkage (N- or O-linked), position, or structural composition of the glycans, suggesting a possible generality of these effects in glycoproteins. Additionally, these studies also provided several explanations for the slightly reduced structural dynamics caused by natural glycosylation, namely, restriction of the protein conformational sampling space by steric crowding, reduction of solvent access to the protein surface, and direct hydrogen bonding contacts between the protein surface and the first surface accessible residues of the glycans [16, 31, 53–55].

Although these results from naturally glycosylated proteins highlight the connection between glycosylation and the changes in overall protein structural dynamics, several fundamental issues still remained unaddressed (e.g., Is the magnitude of the changes in structural dynamics influenced by the protein glycan content or the size of the attached glycan? and Is this a general phenomenon achievable by the chemical attachment of non-natural glycans to the protein structure?). To address these questions, Solá and Griebenow recently performed a series of studies in which they employed chemical glycosylation to systematically explore the influence that alterations in the glycosylation parameters had on the overall biophysical properties of the serine protease α-chymotrypsin (α-CT) [30, 56, 57]. α-CT was chosen as a model protein since its inherent biophysical properties were already well characterized and understood. For these studies, series of α-CT glycoforms (Fig. 1) were created with systematic variations in the amount of glycan bound to the protein surface and in the glycan size through the use of chemically activated glycans of differing molecular masses [Lac: lactose (500 Da); Dex: dextran (10,000 Da)]. The glycans were covalently attached to the protein via the reactivity afforded by the surface amine groups (amino terminus and lysine residues) towards a succinimidyl functionalized linker [58]. Upon characterization of the changes in structure and dynamics of the different α-CT glycoconjugates by circular dichroism (CD) spectroscopy and FTIR (Fourier transform infrared) amide H/D exchange experiments, they arrived at the following conclusions concerning the effects of chemical glycosylation (Fig. 2): a) increases in the size and amount of chemically attached glycans do not alter the original structural fold of the protein, b) a substantial decrease in protein structural dynamics could be systematically induced through chemical glycosylation, and c) this reduction in structural dynamics only depends on the amount of glycans bound to the protein surface and not on the glycan size. The similarity between these results derived from chemical glycosylation and those from natural glycosylation thus seemed to confirm the general nature of these effects by glycans. This prompted the researchers to hypothesize about the potential utility of chemical glycosylation for the systematic perturbation of protein structural dynamics within biochemical applications [30, 57] (some examples will be discussed further on).

Influence of glycosylation on protein thermodynamics

Another fundamental biophysical property which becomes altered upon natural glycosylation is the thermodynamic stability [13]. Although several researchers had previously highlighted differences in stability for naturally glycosylated proteins [38, 47, 59], the first detailed biophysical studies attributing the increase in thermostability found for glycoproteins to the glycan moiety were described by Wang et al. in 1996 [60]. In this work a comparative calorimetric study on five naturally glycosylated proteins (yeast external invertase, bovine serum fetuin, glucoamylase from *Aspergillus niger*, chicken egg white ovotransferrin, and avidin) was performed. In all five cases, small decreases in the denaturation temperatures (T_m), denaturation enthalpies (ΔH_m), and thermal unfolding reversibilities were found after enzymatic deglycosylation [60]. The magnitude of these effects was independent of the linkage and branching of the glycans but depended on the carbohydrate content of the structurally different glycoproteins. This again supports the view of a general nature to these effects. It was also concluded that the glycan moiety not only increases the thermodynamic stability of glycoproteins but also alters their folding equilibria, possibly through decreased protein aggregation in the unfolded state [60]. In agreement with these findings, Mer et al. (1996) found an increase of 1 kcal/mol in thermodynamic stability for the glycosylated isoform of the proteinase inhibitor PMP-C through calorimetric studies [52]. Subsequent comparative calorimetric studies between the glycosylated isoform of chicken ovomucoid first domain and its non-glycosylated isoform led DeKoster and Robertson (1997) to con-

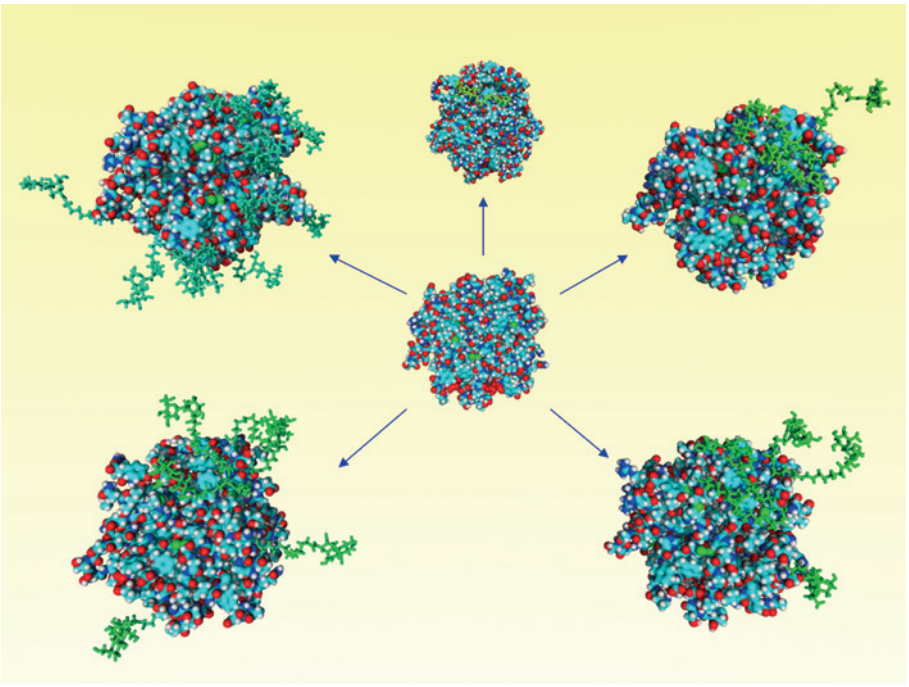

Figure 1. Molecular structures for the α-chymotrypsin (α-CT) glycoconjugates engineered through chemical glycosylation. α-CT (center) and α-CT glycoconjugates with glycosylation degree increasing clockwise from top (Lac_n-α-CT with varied n: 1, 3, 5, 7, and 14). Protein presented in CPK style with standard atom coloring, glycans presented in stick style with green coloring: Models adapted from [57] with permission. The image was created with YASARA (www.yasara.org).

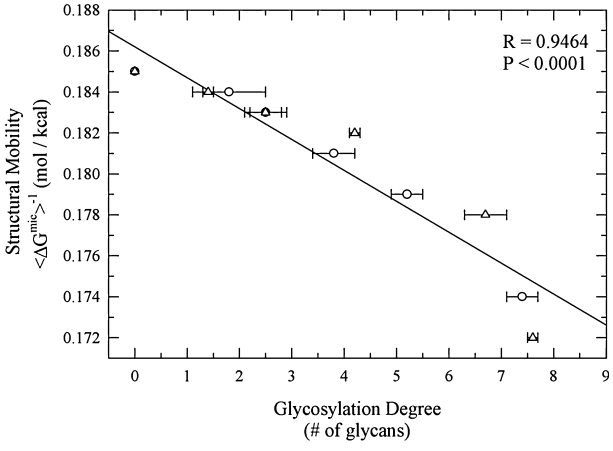

Figure 2. Changes in protein structural mobility ($\langle \Delta G^{mic} \rangle^{-1}$) as a function of glycosylation degree and glycan size [lactose (○) and dextran (Δ)] for the various α-CT glycoconjugates engineered through chemical glycosylation. Glycosylation degree is equal to the average number of glycan molecules chemically attached to the protein surface. Reproduced from [30].

clude that the increase in thermodynamic stability of glycoproteins was mainly of an entropic nature [61]. Another study that provided some additional fundamental insights into the causes of the increased in thermodynamic stability of glycoproteins was performed by Kwon and Yu in 1997 [62]. Here, the effects of glycosylation on the unfolding and refolding rates of human alpha 1-antitrypsin were studied by urea-induced equilibrium unfolding experiments.

Through kinetic analysis it was found that glycosylation slows the unfolding process without affecting the refolding rates significantly. From these results it was proposed that the increase in thermodynamic stability caused by glycosylation could be due to stabilization of the native state and not due to destabilization of the unfolded state [62] (the mechanistic causes of this phenomena will be explained in more detail below). In agreement with these results, Leong and Middelberg recently reported that the refolding process of alpha-fetoprotein (AFP) occurs independent of its glycosylation state [63]. However, while these results suggest that protein folding is not influenced by glycosylation, several other research groups found refolding to be promoted by the presence of the glycans. For example, Yamagushi and collaborators recently ascribed a chaperone-like function to N-linked glycans due to their direct promotion of polypeptide folding [64–66]. Additionally, recent studies performed by Surolia and collaborators on several members of the lectin family have revealed that the presence of the glycan moiety for this class of glycoproteins can alter the folding-unfolding pathway as well as its intermediates [67–69]. The differences in results in these studies suggest that these effects may be of a protein dependent nature. Therefore, more studies are still necessary to fully understand the nature of glycan-promoted protein folding and the changes to folding pathways (for recent developments in this area see the work of Imperiali and collaborators [70]).

While these results derived by using naturally glycosylated proteins suggest a connection between glycosylation and increased glycoprotein thermostability, several issues still remained unaddressed (e.g., How

do the glycosylation parameters influence the magnitude of changes in thermostability? and Is this also a general phenomenon achievable by the chemical attachment of non-natural glycans?). Through the use of chemical glycosylation with small sized glycans (e.g. glucose, fructose), De Jongh and collaborators recently reported that beta-lactoglobulin thermostability could be artificially enhanced by increasing the degree of glycosylation, suggesting the generality of these effects [71]. It was proposed that glycans could achieve such effects by lowering the protein's change in heat capacity of unfolding [$\Delta Cp = Cp(unfolded) - Cp(native)$] possibly through a mechanism which involves the association of the exposed non-polar residues in the unfolded state with the glycans, thereby preventing their solvent exposure [72]. To determine the influence that the glycosylation parameters have on increasing the thermodynamic stability of proteins, Solá et al. recently performed a detailed thermodynamic study on the chemically glycosylated α-CT conjugates through the use of differential scanning calorimetry (DSC) [30, 56]. This investigation revealed the following details about protein thermal stabilization by glycans: a) increases in the glycosylation degree can shift the melting temperature (T_m) linearly to higher temperatures independent of the glycan molecular size (e.g., up by 7 °C for seven bound glycans) (Fig. 3a), b) the change in heat capacity of unfolding (ΔCp) decreases with increases in the glycosylation degree (from 6.2 kcal/mol K to 3.9 kcal/mol K) (Fig. 3b), c) increases in glycan size lead to a more pronounced lowering of ΔCp (from 6.2 kcal/mol K to –2.1 kcal/mol K) reaching even negative values for the most glycosylated conjugates, which is rare for protein unfolding (Fig. 3b), and d) the Gibbs free energy of unfolding at 25 °C [$\Delta G_U(25^oC)$] for α-CT [which is indicative of overall protein stability (thermodynamic, kinetic, and colloidal)] increased with increases in the glycosylation degree (from 5.5 to 8.7 kcal/mol) and to an even larger extent with increases in glycan size (from 5.5 to 13.3 kcal/mol) (Fig. 3c). These results show that glycosylation parameters can play different roles in the overall stabilization of proteins (e.g., glycosylation degree mainly influences protein thermal stability, while glycan size influences the kinetic and colloidal stabilities). The mechanistic basis of these phenomena will be discussed in more detail further on.

Influence of glycosylation on enzymatic catalysis

One of the most general statements concerning the modulation of protein biophysical properties by glycosylation is related to its effect on altering protein function. Ironically, this has remained one of the least-understood areas in glycoprotein biophysics due to the few detailed mechanistic studies performed. To simplify the discussion, we will limit the following section to those *in vitro* reports on glycoproteins which possess enzymatic function. One of the earliest examinations of the role of glycosylation on the enzymatic function of a glycoprotein was performed by Grinnell et al. in 1991 while studying the catalytic behavior of the different glycoforms of the antithrombotic serine protease human protein C (HPC) [73]. Upon sequential elimination of the enzyme's glycosylation sites by site-directed mutagenesis, they found an increase in the catalytic rates (k_{cat}) without apparent changes in substrate binding affinities (K_M). These pioneering results prompted the researchers to hypothesize that the catalytic efficiency of the enzyme could possibly be tuned through the use of carbohydrate modifications [73], a phenomenon that was recently evidenced by Solá and Griebenow on a structurally homologous serine protease (α-CT) through the use of chemical glycosylation (see details below) [30]. Similar results were also obtained by Dwek and collaborators while studying the effects of natural glycosylation on the function of the different RNase, plasminogen, and tissue plasminogen activator glycoforms [47, 49, 74, 75]. In these studies a reduction in the enzymatic activity for the three different glycoproteins was observed, with the changes being mainly reflected in the catalytic rates (k_{cat}). Klinman and collaborators also found a decrease in the catalytic rates without apparent changes in substrate binding affinity for the reaction catalyzed by the different glycosylated isoforms of glucose oxidase [76].

Through the use of chemical glycosylation Solá and Griebenow recently examined the influence of variations in the glycosylation parameters on the kinetics of the peptide hydrolysis reaction catalyzed by the serine protease α-CT [30, 57]. They arrived at the following conclusions concerning the effects of glycosylation on enzymatic catalysis: a) increases in the size and the amount of the chemically attached glycans did not alter the substrate binding affinity (K_M, K_S), b) chemical glycosylation induced a significant reduction of the catalytic rates (k_{cat}, k_2, and k_3), and c) this reduction only depended on the amount of glycans bound to the protein surface being independent of the glycan size (Fig. 7; lower right panel). Interestingly, if the changes in enzymatic activity induced by both natural and chemical glycosylation are qualitatively compared, it becomes clear that they are quite similar, even though these were obtained with structurally and functionally different proteins. This suggests that these effects by glycans may be of a general nature, possibly serving as a mechanism to regulate protein function *in vivo*.

Biochemical insights through chemical glycosylation

Novel insights into the structurally dynamic nature of protein biophysics

Understanding the interrelationships between the fundamental biophysical properties of proteins (e.g., structure, dynamics, thermostability, and function) is essential for predicting and controlling their behavior within biochemical and biomedical applications. Although a large number of past studies regarding protein biophysical behavior have focused on the classical 'static' view of proteins, an alternative 'dynamic' view has developed in recent years due to the intrinsically dynamic nature of the protein structure [77]. While the direct connection between changes in protein structure and its effects on protein biophysical properties has been long encompassed in the so-called structure-stability and structure-function relationships [78], the influence of structural dynamics within these relationships, although long envisioned [79–92], has remained statistically unexplored due to the lack of effective chemical strategies for the systematic perturbation of protein structural dynamics. The importance of designing chemical strategies for the effective control of protein structural dynamics to answer this and other biochemically related fundamental questions is such that it has been identified by a series of leading scientific researchers (including several Nobel laureates) in a recent survey published in *Nature* as one of the six most important biochemical research problems expected to be addressed by chemists [93].

While studying the possible mechanistic causes for altered protein structural dynamics, thermodynamic stability, and enzyme kinetics induced through chemical glycosylation, Solá and Griebenow realized that these effects could be interrelated by invoking a dynamic connection between the structure and the biophysical properties of glycoproteins [30], as had been previously invoked for mesophilic-thermophilic homologous protein pairs by Petsko and collaborators [94] and for phosphorylated proteins by Kern and collaborators [95]. By performing a statistical correlation analysis of the changes in protein properties induced by chemical glycosylation, Solá and Griebenow were able to statistically confirm the existence of an inverse relationship between the changes in protein structural dynamics and the changes in protein thermodynamic stability (Fig. 4a). This observation of an inverse relationship between protein structural dynamics and thermodynamic stability thus evidences the direct dependence of global protein stability on the enthalpic (molecular interactions) and entropic (molecular dynamics) components of the protein structure, as previously proposed by Privalov and Tsalkova [79]. Any decrease in protein structural dynamics should therefore lead to a smaller contribution of the protein's internal entropy to its overall stability, thus shifting the thermodynamic unfolding equilibrium towards higher T_m values (Fig. 3a). These arguments are in complete agreement with the notion of an entropically induced increase in thermodynamic stability for glycoproteins as has been proposed by DeKoster and Robertson [61].

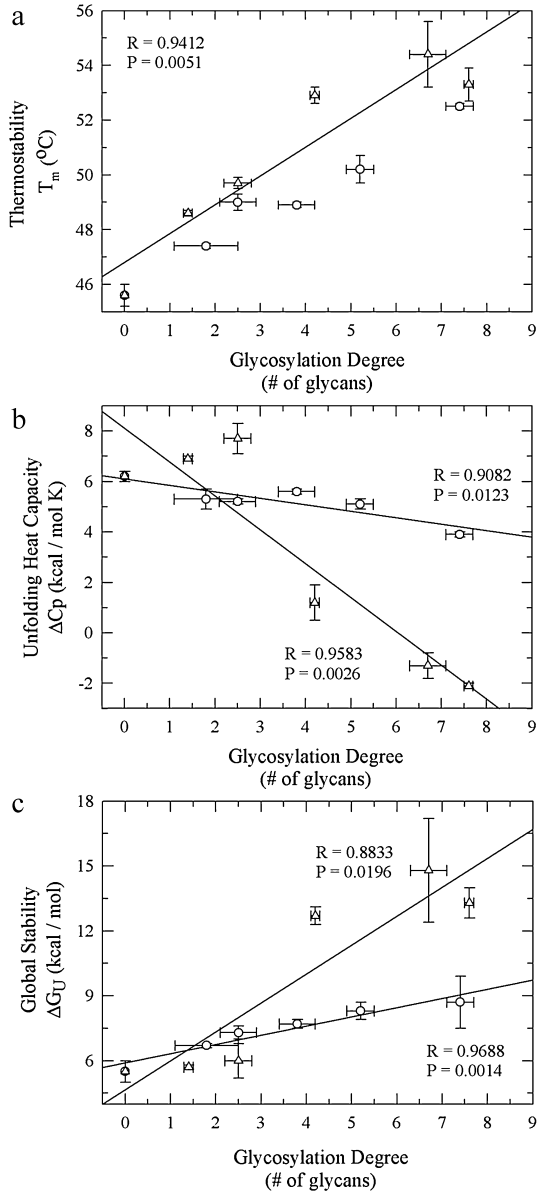

Figure 3. Changes in thermodynamic unfolding parameters as a function of glycosylation degree and glycan size [lactose (○) and dextran (Δ)] for the various α-CT glycoconjugates engineered through chemical glycosylation. Adapted from [56] with permission.

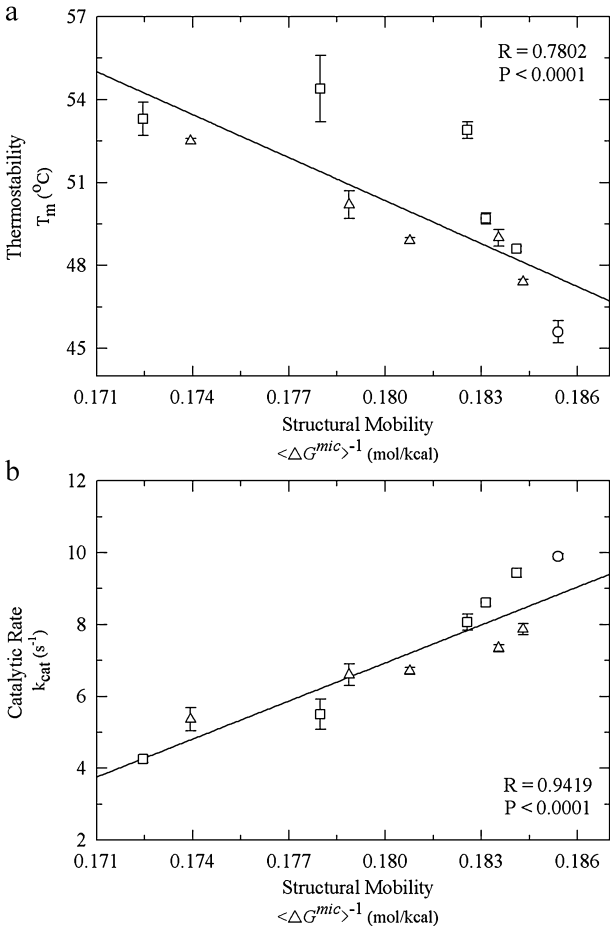

Figure 4. Statistical correlation analysis (ANOVA) between changes in global structural mobility ($\langle \Delta G^{mic} \rangle^{-1}$) and changes in thermodynamic stability (T_m) and catalytic activity (k_{cat}) for α-CT (○) and for the various Lac-α-CT (Δ) and Dex-α-CT (□) glycoconjugates engineered through chemical glycosylation. Reproduced from [30].

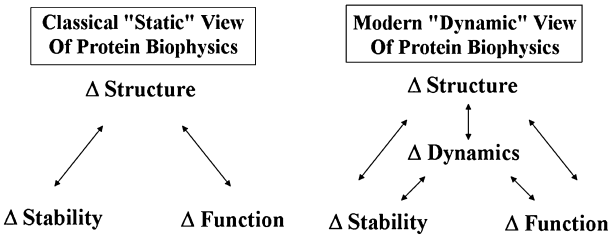

Figure 5. Schematic diagram displaying the classical 'static' view of protein biophysics, where the changes in the protein biophysical behavior are due to conformational changes in the protein structure, and the modern 'dynamic' view of protein biophysics confirmed through chemical glycosylation [30], where the changes in protein biophysical behavior are additionally influenced by changes in the velocity of the conformational changes in the protein structure.

Through statistical analysis it was also found that a direct relationship between the changes in protein structural dynamics and enzyme catalysis exists while under similar experimental conditions (e.g., internal amino acid composition, pH, temperature, and solvent) (Fig. 4b) [30]. This observation indicated that as previously hypothesized for α-CT [96–98] and for enzyme systems in general [99–102], the dynamical energy of the enzyme could be transduced towards the chemical steps during catalysis, thereby impacting the reaction's transition-state Gibbs free energy of activation. These observations derived through the use of chemical glycosylation have therefore provided for the first time statistical support to the modern 'dynamic' view of protein biophysics (Fig. 5) where the changes in protein biophysical properties depend not only on the structure but also on its dynamics. While for the classical 'static' view of protein biophysics the changes in protein stability and function have been largely explained by the magnitude of conformational changes [structural displacements (Å)], in the modern dynamic view of protein biophysics these changes are now additionally influenced by changes in the magnitude of the speed [structural velocity (Å/μsec)] with which the protein samples through these different conformations (Fig. 5). The statistical validation of this novel perspective on protein biophysics will thus have direct implications for the future mechanistic understanding of several fundamental biochemically related processes, such as enzymatic catalysis [87, 88, 91, 100, 102–107], its allosteric regulation [95, 108–113], structural cooperativity effects [77, 114], and enzyme stability engineering [115].

Novel mechanistic insights into glycoprotein biophysics via computational methods

To provide further mechanistic insights into the nature of the aforementioned changes in glycoprotein biophysics, Solá and Griebenow recently performed a detailed computational study to explore the effects that the degree of glycosylation had on the conformational energetics and dynamics of the α-CT glycoconjugates [57]. For these studies, the reactivity profiles of α-CT's lysine residues were calculated, and *in silico* molecular models of the glycoconjugates constructed with increasing amounts of glycans attached to the protein's surface (Fig. 1) by employing the YASARA suite of molecular modelling programs (YASARA Biosciences, Graz, Austria) [57]. Accurate energetic equilibration of the glycoconjugate structures was performed by quantum mechanical parameterization of the glycans with YASARA's AutoSMILES protocol [116] and by employing the AMBER [117] and GLYCAM [118] molecular mechanics force field parameters derived for glycoproteins coupled with rounds of molecular dynamics (MD) simulations. These modelling studies revealed that as the amount

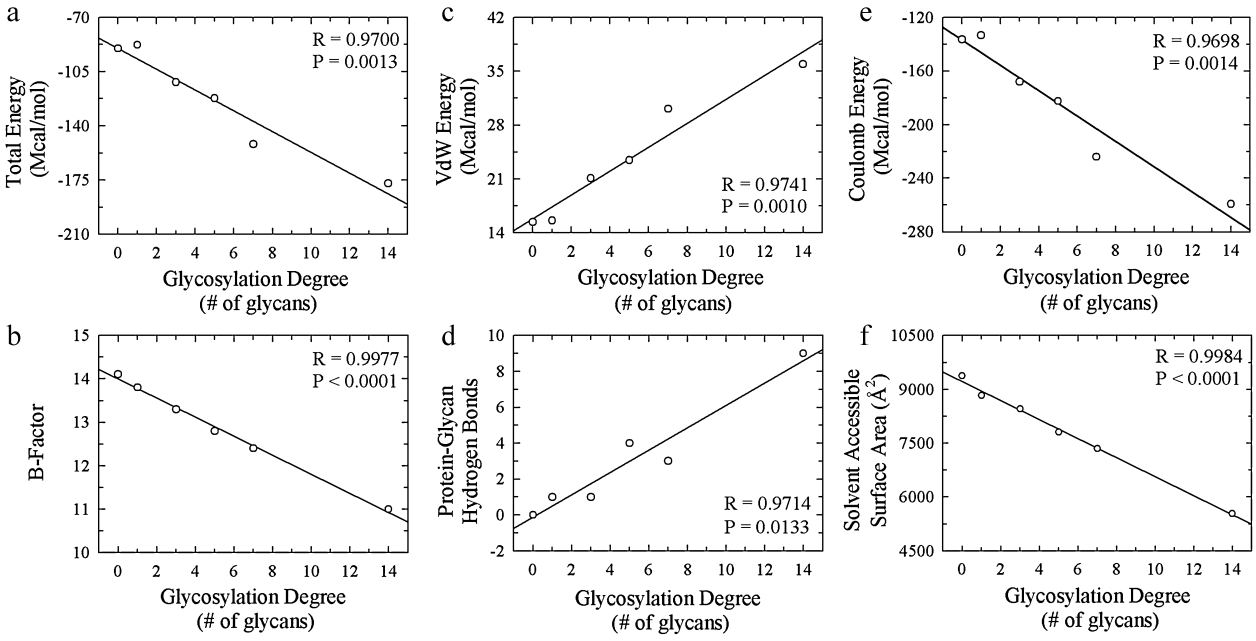

Figure 6. Changes in energetic (total, VdW, coulombic), entropic (B-factor), hydrogen bonding, and solvent-accessible surface area parameters as a function of glycosylation degree derived from the molecular models of the various α-CT glycoconjugates (see Fig. 1). Glycosylation degree is equal to the number of glycan molecules chemically attached to the protein surface. Adapted from [57] with permission.

of glycosylation increased, the values for the total internal energy parameter and global Debye-Waller temperature B-factor (which is a crystallographic measure of structural entropy and dynamics [119]) for the glycoconjugate structures decreased (Fig. 6a,b) [57]. These results indicate that at increasing glycosylation degree the protein structure becomes both energetically more stable and rigid. Interestingly, the changes between the experimental and theoretical energetic (T_m vs. total energy) and dynamic (ΔG_{HX} vs. B-factor values) parameters determined for these glycoconjugates correlated statistically (ANOVA; $R > 0.90$, $P < 0.025$), which suggests that the *in silico* models constructed accurately reflected the experimental behavior of the glycoconjugates.

To mechanistically explain the nature of the decreased structural dynamics and internal energetics for the glycoconjugates, Solá and Griebenow further examined the effects of glycosylation on the individual energetic terms for the non-covalent forces arising within the protein peptide core [57]. It was found that as glycosylation increased, the value for the coulombic energy parameter substantially decreased with a concomitant increase in internal van der Waals (VdW) energy for the protein portion of the conjugates (Fig. 6c,e). This indicates that glycosylation can lead to an overall more stable and rigid protein structure due to increased internal electrostatic attractions coupled with a greater number of hydrophobic packing contacts. Interestingly, the importance of internal electrostatics on influencing the dynamics and stability of proteins has also been noted by Affleck et al. [120], Simonson [121], and by Strickler et al. [122] in other protein systems. In looking for a physical explanation for the nature of these effects by glycans, Solá and Griebenow first evaluated the changes in the hydrogen bond composition of the glycoconjugate models (Fig. 6d). Only a few new hydrogen bonds formed between the protein surface and the glycan moieties, thus disproving the hypothesis of changes in glycoprotein behavior due to direct hydrogen bonding contacts between the protein surface and the first surface accessible residues of the glycans. The changes in solvent-accessible surface area upon glycosylation for the glycoconjugate models were examined next. Here, it was found that although the total solvent-accessible surface area for the glycoconjugates increased significantly with increased amounts of glycosylation (9,000 Å^2 to 16,000 Å^2), the surface exposed area for the protein portion of the conjugates was actually significantly decreased (9,000 Å^2 to 5,000 Å^2) (Fig. 6f). This result highlights the importance of the solvent (e.g., aqueous media vs. organic solvent) and its contact with the protein matrix in influencing the internal electrostatics and biophysical properties of proteins.

This raises the question of how a decrease in solvent-accessible surface area due to glycosylation can lead to an increase in protein internal electrostatic attractions

and structural rigidification. Water, being a highly polar solvent ($\varepsilon = 78.3$; dielectric constant), exhibits both a high dielectric susceptibility and highly fluctuating electric dipole moment [123, 124], affecting the protein electrostatics in several ways. The high dielectric susceptibility makes water very sensitive to polarization when an external field is applied (e.g., long-range protein charge-charge interactions), leading to diminished protein electrostatic interactions due to charge screening [125, 126]. The highly fluctuating electric dipole moment of water increases the fluctuations in the outer dipole layers of the protein (those that interact with the hydration water dipoles) [123, 124, 126], decreasing the protein internal dipole-dipole interactions and increasing protein structural vibrations [120]. Removal of hydration water from the protein surface due to glycosylation should reverse both of these phenomena, thereby increasing protein internal electrostatic attractions and decreasing protein structural dynamics. Thus, glycosylation can be envisioned as leading to decreased contacts between the protein electrostatics and the solvent electrostatics due to 'steric dielectric shielding', which would transform the protein biophysical properties from being solvent slaved to non-slaved [123, 124] (slaved refers to molecular phenomena influenced by the solvent electric dipole moment fluctuations). This phenomena has such wide-reaching implications that it has been noted by several researchers as one of the principal causes for the decreased activities exhibited by enzymes when employed as biocatalysts within organic media [120, 127–134].

From these computational and experimental studies Solá and Griebenow [30, 56, 57] have now provided an integrated mechanistic perspective of the changes in glycoprotein conformational energetics and dynamics caused by increasing the glycosylation degree (Fig. 7). Increases in the glycosylation degree would first reduce the protein solvent accessible surface area by sterically shielding the surface of the protein (a concept that was originally proposed by Dwek and collaborators [46]). This would prevent the solvent-electrostatics from interacting with the protein electrostatics, thereby increasing the internal electrostatic attractions and VdW interactions of the protein. This increase in internal non-covalent binding forces (an enthalpy loss) would simultaneously lead to a decrease in protein structural dynamics (an entropy loss) in accord with the concept of entropy-enthalpy compensation in proteins [135–137]. Both of these enthalpic and entropic changes must also impact the natively folded state, thereby increasing the protein's thermodynamic stability. The decrease in protein structural dynamics, reflected in dampened domain motions, will also lead to a decrease in enzymatic activity due to structural mechanochemical coupling (see next section) [30]. Interestingly, a similar connection between decreased surface hydration and loss of α-CT activity was also recently highlighted by Zewail and collaborators through the use of femtosecond spectroscopy [138]. The discovery of this novel mechanism of steric dielectric shielding by a chemical modification such as glycosylation could have profound implications for the design of novel protein stabilization strategies, since these effects in principle should be achieved by other types of chemical modifications which can displace large volumes of solvation water from the protein surface or that can alter protein internal electrostatics (a recent example validating this concept will be discussed below).

Novel mechanistic insights on the structurally dynamic nature of protease catalysis

Deciphering the general mechanisms by which enzymes accelerate chemical reactions is fundamental to the understanding of biochemical processes. Although the chemical nature of enzyme catalysis is in large parts understood [139], there is still some controversy regarding the contribution of concepts such as the involvement of protein structural dynamics in the chemical steps of catalysis (e.g., via mechanochemical coupling between global dynamics and active-site chemical groups) [100, 103, 140–144]. Having validated the potential of chemical glycosylation as a tool for the systematic perturbation of protein structural dynamics [30], Solá and Griebenow recently employed it to perform a detailed study on the contribution of structural dynamics to the individual steps of the catalytic mechanism of chymotrypsin-fold serine proteases [57]. Although the chemical components involved in the catalytic mechanism of this biochemically and biomedically important class of enzymes (members include thrombin, several coagulation and complement factors, the trypsins, the kallikreins, and the granzymes) are some of the best understood (e.g., the His_{57}-Asp_{102}-Ser_{195} catalytic triad), the influence of structural dynamics within their catalytic mechanism has remained largely unexplored [97].

Being the representative member of the structural clan of chymotrypsin-fold serine peptidases [Clan PA (Family S1); MEROPS Database] [145], α-CT catalyzes the selective hydrolysis of amide bonds adjacent to bulky hydrophobic side chains from its peptidic and proteic substrates. Its catalytic cycle (Fig. 7; lower right panel) is composed of a primary non-covalent substrate binding step (K_S) followed by two chemical steps which involve the acylation (k_2) and deacylation (k_3) of the enzyme with the formation of two acyl-enzyme tetrahedral intermediates (TET1 and TET2). By

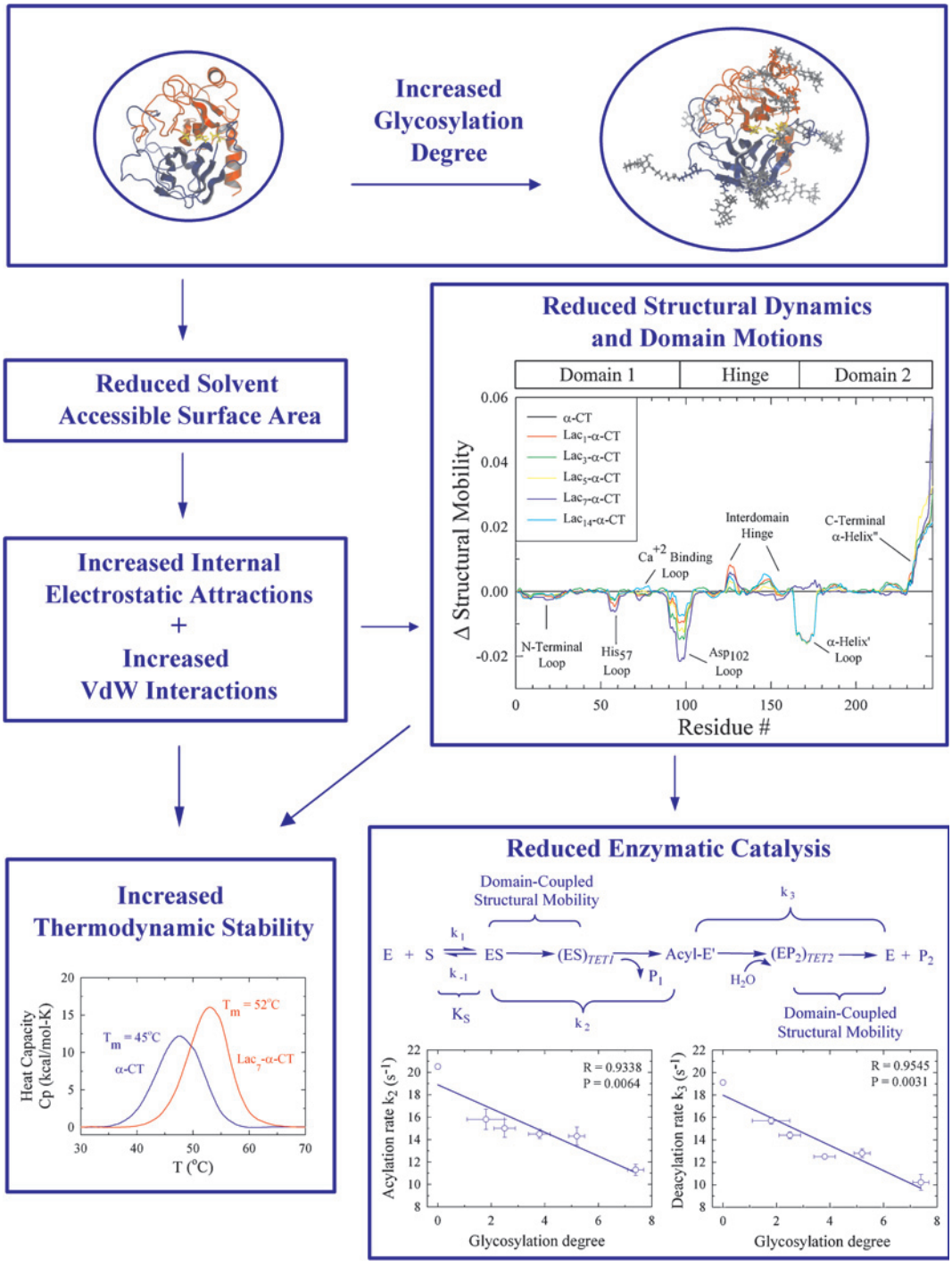

Figure 7. Integrated mechanistic perspective of the changes in protein biophysical properties induced through increases in glycosylation degree. Portions of the figure were adapted from [56, 57] with permission. Image partially created with YASARA (www.yasara.org).

performing amide H/D exchange and chemical kinetic dissection experiments for the chemically glycosylated α-CT conjugates, Solá and Griebenow were able to show that changes in protein structural dynamics impacted both acylation (k_2) and deacylation (k_3) rates without altering the binding of the substrate (K_S) (Fig. 7; lower right panel) [57]. Interestingly, the activation energy for both chemical steps increased linearly as the structural dynamics of the enzyme were reduced through chemical glycosylation [57]. While this result supported the notion of dynamical energy transduction within the mechanism of serine protease catalysis, it did not provide for a structural mechanistic explanation of this phenomenon.

Since this class of proteases is structurally composed of two six-stranded Greek-key β-barrel domains (Fig. 7), the nature of their collective structural dynamics has been mainly ascribed to interdomain hinge-bending motions [97, 146, 147]. Due to the location of the active-site residues at the interface between these two domains, it had been previously suggested that the global structural flexibility of the enzyme could directly influence their structural displacements, therefore potentially impacting the reaction kinetics [97, 147–149]. To verify the validity of this hypothesis, Solá and Griebenow submitted their energy equilibrated α-CT glycoconjugate models to Gaussian network model (GNM) computational analysis [57]. The Gaussian network model represents a simplified version of normal mode analysis for describing the fluctuations in dynamics of polymer networks. This type of structural analysis was developed to provide a simple and computationally inexpensive yet accurate description of residue interconnections and mobilities within the collective vibrational modes of proteins and supramolecular structures [150, 151]. Results from this type of calculation have been found to be in excellent agreement with experimental X-ray crystallographic B-factors, H/D exchange free energies of amide protons, and NMR-relaxation order parameters [152, 153]. Due to this the GNM has been used extensively to describe the influence of collective structural motions on the functional behavior of proteins. Analysis of the collective dynamics of α-CT by this methodology revealed that protein structure is characterized by two relatively rigid domains [domain 1 (residues 1–119) and domain 2 (residues 151–245)] connected by an interdomain hinge (residues 120–150) (Fig. 7) [57]. The connection between these two domains and the hinge occurs via two highly mobile loops [Asp_{102} loop (residues 85–105) and the α-helix′ loop (residues 160–175)] located at the structural edges of each domain whose dynamics give rise to the characteristic domain hinge-bending motions of this structural class of protease. When GNM analysis was applied to the α-CT glycoconjugates (Fig. 7), the relative structural mobilities of the Asp_{102} loop and the α-helix′ loop as well as that of the His_{57} loop were reduced at increasing levels of glycosylation. This result indicated that glycosylation could lead to a reduction in the kinetics of the protein domain motions without altering the shape of the collective vibrational mode [57]. This decrease in mobility for the His_{57} and Asp_{102} loops thus provided a mechanistic explanation for the decreased acylation and deacylation rates observed after chemical glycosylation, as these residues are directly involved in the hydrogen transfer steps necessary for catalysis. A decrease in the dynamics of these two loops would reinforce the hydrogen bonding strength between the His_{57} and Asp_{102} residues, leading to a possible anticatalytic situation via the permanent formation of a low-barrier hydrogen bond as proposed by Ishida [154]. This would affect the deprotonation and protonation rates of Ser_{195}, thus reducing the kinetics of catalysis [57]. By systematically perturbating α-CT's structural dynamics through chemical glycosylation [57], Solá and Griebenow have now identified the structural mechanism by which mechanochemical coupling between domain motions and active-site chemistry [106] occurs within the catalytic mechanism of chymotrypsin-fold serine proteases [96–98, 147]. Interestingly, a recent study conducted by Carnevale et al. on the representative members of the protease superfamily identified the conservation of such large-scale motions across members with differing structural folds and active-site chemistries, suggesting that the phenomenon of mechanochemical coupling at least for the protease superfamily may be of an evolutionary nature [155]. These experiments by Solá and Griebenow [30, 57] therefore highlight the potential utility of chemical glycosylation as a tool for the mechanistic understanding of protein biochemical and biophysical phenomena, especially those influenced by protein structural dynamics.

Biomedical implications of chemical glycosylation

Improving protein long-term stability within therapeutic applications

The employment of proteins as pharmaceutical agents has redefined the field of therapeutic pharmacology, with hundreds of protein molecules currently approved for therapeutic use and many more being in the process of discovery and formulation [156]. Yet, as the number of biotherapeutics being developed increases, so does the general knowledge about the fundamental physicochemical instability issues that appear to plague these macromolecules [157–162]. In contrast to traditional small-molecule therapeutics, whose physicochemical stabilities are much simpler to predict and control [163], the complexities arising due to the supramolecular nature of protein structure burden the development of predictive methodologies concerning their chemical as well as physical stabilization [164]. To grasp the complexities concerning the issue of protein stability, one needs to take into consideration the nature of and the interrelations between the different levels of protein structure and architecture which arise due to the intra- and intermolecular interactions between the various amino acid building blocks of the protein and how these

interactions become perturbed depending on the physicochemical environment to which the protein becomes exposed. While the protein primary structure is subject to the same physicochemical instability issues as traditional small-molecule therapeutics (e.g., acid-base and redox chemistries, chemical fragmentation, deamidation, etc.) [165], the higher levels of protein structure (secondary, tertiary) which are requisite for protein function display lower levels of thermodynamic stability (2–5 kcal/mol for tertiary structure vs. 10–25 kcal/mol for the most labile chemical bonds) due to their non-covalent nature thus giving rise to irreversible structural changes, unfolding, and subsequent loss of function (kinetic inactivation) observed in protein macromolecules even at relatively low temperature values (20–40 °C) [166]. Additionally, due to their colloidal nature these macromolecules display pH-, temperature-, and concentration-dependent non-native supramolecular aggregation which has been linked to life-threatening immunological reactions and poses one of the biggest challenges for the successful employment of biotherapeutics [167, 168]. Due to these instability issues much emphasis has been given to the development of strategies for the effective stabilization of proteins [79, 115, 160, 166, 169].

In general, protein stability can be either increased internally by altering the chemical composition of the protein, or externally by modulating the properties of the surrounding solvent [115, 160, 166]. While much emphasis has been given to external protein stabilization by the use of co-dissolving additives, their use can be problematic. This is in large part due to their concentration- and protein-dependent nature, which often demands the employment of high molar excesses of these reagents to achieve even modest thermodynamic stabilization effects ($\Delta Tm = 1\ ^{o}C$) [160]. Their practical use for drug delivery purposes can also become limited, as they often leach out of the drug delivery matrix during device manufacturing [159]. Excipients can additionally cross-react with the multiple chemical functionalities present in the protein, thus leading to further stability problems in the long run [160, 161]. Due to these problems, alternative strategies involving internal protein stabilization via structural modification are currently being explored (e.g., site-directed mutagenesis, formation of novel disulfide bonds, etc.). Amongst them, covalent chemical modification of the protein surface represents one of the most promising approaches for the stabilization of proteins during their use in industrial and pharmaceutical applications, as it is generally perceived that through manipulation of key parameters (e.g., modification degree, chemical composition, and size of the modifier) the protein's biophysical properties could be engineered as desired [158, 170]. In this context, chemical glycosylation represents a promising methodology to modulate protein long-term stability under pharmaceutically relevant conditions [158].

The first hypothesis on the potential kinetic stabilization of proteins by glycosylation was reported by Vegarud and Christnsen in *Biotechnology and Bioengineering* in 1975 [59], after realizing that glycoproteins were resistant to proteolytic inactivation [171]. The same year Holcenberg et al. [172] reported increased blood plasma half-lives for asparaginase after glycosylation. Subsequent experimental studies by Dellacherie et al. (1983) [173], Lenders and Chricton (1984) [174], and Srivastava (1991) [175] evidenced an increase in the functional lifetimes (kinetic stability) of hemoglobin and amylase when exposed to high temperatures upon cross-linking with different soluble polysaccharides. In 1995 Baudys et al. reported that the physical stability of insulin could be improved by reducing its aggregation kinetics through the chemical attachment of small sized glycans [176]. These reports were followed by those of Hoiberg-Nielsen et al. (2006) in which the thermodynamic, kinetic, and colloidal stabilities for the glycosylated and non-glycosylated forms of *Peniophora lycii* phytase were compared by calorimetric as well light-scattering methods [177]. It was found that the presence of the glycans significantly increased the kinetic stability by reducing the rate of protein aggregation while leaving the equilibrium melting temperature largely unaltered. It was suggested that the inhibition of aggregation was likely dependent on steric hindrance of the glycans in the unfolded protein state [177] and not on their hydration properties [178, 179].

While all of these studies highlighted the potential application of glycosylation for the modulation of protein long-term stability there was still a lack of fundamental knowledge regarding the mechanisms by which it could lead to improvement in protein stability within therapeutic applications since the effects of the different glycosylation parameters had not been systematically explored. To further the development of glycosylation for the stabilization of protein therapeutics, Solá et al. recently conducted a detailed study directed at understanding the mechanisms by which systematic changes in the glycosylation parameters could alter protein thermodynamic, kinetic, and colloidal stability [56]. As already described in a preceding section, increases in the glycosylation degree can lead to an increase in protein thermostability (T_m) independent of glycan size although overall stability [$\Delta G(25\ ^{o}C)$] becomes significantly improved with increasing glycan molecular size (Fig. 3). These results by Solá et al. [56] coupled with

those of Hoiberg-Nielsen et al. [177] are intriguing. They highlight the fact that protein samples with similar thermal stabilities (T_m values) will not necessarily have similar overall stabilities (which is often an assumption in the study of protein stability). These results suggest that both glycosylation degree and glycan size should play different roles in the overall stabilization of proteins, quite possibly by influencing protein thermodynamic, kinetic, and colloidal stability through different mechanisms.

When the effects of glycosylation on the kinetics of inactivation were studied under accelerated stability conditions, both the degree of glycosylation and glycan size were found to increase protein inactivation half-lives (kinetic stability), but with the larger-sized glycan leading to a doubling in the magnitude of the inactivation half-lives when compared to the small-sized glycan [56]. Here we want to point out that although the mechanism by which α-CT becomes inactivated could be related to protection against possible autoproteolytic cleavage, we found that this was not the case. Similar fragmentation patterns were found to occur for the protein at pH values where the protein was both catalytically active (pH > 5) and inactive (pH < 5) [56]. Structural examination of the protein revealed that it is composed of three polypeptides linked by two structurally crucial disulfide bonds that can become easily chemically reduced (-S-S- > -SH + HS-) upon unfolding, thereby leading to the characteristic fragmentation pattern and its kinetic inactivation. This suggested that glycosylation can increase protein kinetic stability by ameliorating the unfolding and chemical fragmentation of the protein structure [56]. When the effects of glycosylation on the aggregation process were assessed under accelerated aggregation conditions (colloidal stability), it was found that aggregation could not be prevented by the small-sized glycan even at the higher degrees of modification in spite of the fact that the thermal and kinetic stabilities of these samples were vastly improved. In contrast, the aggregation process was completely inhibited by the larger-sized glycan independent of the glycosylation degree.

If one considers a simple two-state model for folded-unfolded protein equilibrium (Fig. 8), it becomes evident that there can be three general mechanisms by which protein overall stability can be increased (either by increasing the stability of the natively folded state, by destabilizing the unfolded state, or by changing the unfolding-folding pathway and its intermediates). Specifically for the case of glycosylation, the stability of the native state is increased by a decrease in the structural dynamics of this state as a result of the increased internal protein electrostatic attractions and VdW forces (Fig. 7). Since the degree of glycosylation was the factor mainly responsible for the decrease in structural dynamics and the increase in thermal stability (T_m) of the glycoconjugates, we can now envision how the degree of glycosylation affects protein overall stability through a mechanism which involves increasing the heat capacity of the native state [Cp(native)] via reduced structural dynamics (Fig. 8a). This increase in Cp(native) in turn would lead to the observed decrease in heat capacity of unfolding [$\Delta Cp = Cp(unfolded) - Cp(native)$] as reported by Solá et al. [56] (Fig. 3b) and Broersen et al. [71, 72]. In contrast, the minimal influence on protein structural dynamics and T_m values by the changes in glycan size suggested that its effect on protein overall stability should alternatively occur by influencing the stability of the unfolded state [56]. This coupled with the vastly improved kinetic and colloidal stabilities thus indicated that glycan size plays a predominant role in decreasing the heat capacity of the unfolded state Cp(unfolded) by destabilizing the protein interactions within this state (possibly through steric effects as noted by Hoiberg-Nielsen et al. [177]) (Fig. 8b) [56]. This decrease in Cp(unfolded) in turn leads to the large decrease in heat capacity of unfolding [$\Delta Cp = Cp(unfolded) - Cp(native)$] observed by Solá et al. [56] when glycan size was increased, since its effects were overlaid on top of that generated by the glycosylation degree (Fig. 3b). These results have therefore provided novel mechanistic insights into different aspects of long-term protein stability caused by changes in glycosylation parameters. Interestingly, these *in vitro* stabilization effects caused by glycosylation can be translated into enhanced stability and increased functional lifetimes for glycosylated proteins *in vivo* [28, 180–185]. This was recently exemplified by the first successful commercial example of glycoengineering conducted on a therapeutic protein by researchers at Amgen. In these studies the blood plasma lifetime of the anemia protein drug erythropoietin alfa (EPO) was improved threefold by creating a hyperglycosylated version of the protein (darbepotin alfa; ARANESP) [28]. These results thus evidence the potential utility of glycosylation for the rational engineering of protein biophysical properties within both *in vitro* and *in vivo* biomedical applications.

Biophysical effects of other biochemically and biomedically relevant chemical modifications

Modulation of protein biophysical behavior by PEGylation

The chemical attachment of synthetic polymer molecules, such as, poly(ethylene) glycol (PEG) to the

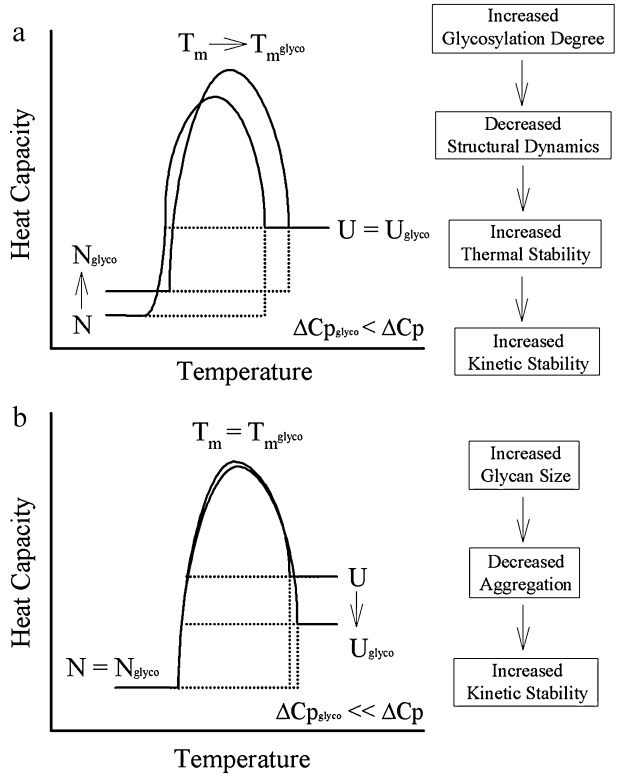

Figure 8. Schematic diagram representing the changes in heat capacity (Cp) as a function of temperature during thermodynamic unfolding for the chemically glycosylated protein conjugates as a function of (*a*) increased glycosylation degree and (*b*) increased glycan size. N and U represent the native and unfolded states for the non-glycosylated protein, while N_{glyco} and U_{glyco} represent the native and unfolded states for the glycosylated protein conjugates. T_m represents the melting transition temperature for the protein, where 50% of the protein is in the native state and 50% is in the unfolded state. Overall change in heat capacity (ΔCp) is equal to the difference in heat capacity between the unfolded and native states [$\Delta CP = Cp(U)\text{-}Cp(N)$].

protein surface (PEGylation) constitutes an increasingly important protein modification of high therapeutic value [186]. Benefits of protein PEGylation include increased stability [187–189], reduced aggregation [190, 191], resistance to proteolysis [188, 192], reduced inmmunogenicity [193, 194], and increased serum half-life [195, 196]. This has resulted in six PEGylated protein formulations (e.g., PEG-asparaginase for cancer treatment and PEG-interferon α2a for hepatitis treatment) currently approved for therapeutic use, with many others undergoing clinical trials [197]. Thus the development of strategies for improving the chemistries of protein PEGylation constitutes an area of increasingly active research [198, 199], with PEGylation chemistries evolving from a 'first generation' of low molecular weight, impure, and unstable reagents to a 'second generation' of reagents with higher purity, a larger range of molecular weights, and chemical modifiers [197, 200].

In spite of all the biomedical studies performed to date on PEGylated proteins focused at improving protein pharmacokinetic and pharmacodynamic behavior [195, 196, 201], there is still a lack of fundamental knowledge regarding the influence of PEGylation on protein biophysical properties. Due to this, Griebenow and collaborators recently conducted a series of studies to address this issue [J. A. Rodríguez-Martínez, R. J. Solá, and K. Griebenow, unpublished observations]. Since the PEGylation chemistry employed in these studies was similar to that used by Solá and Griebenow for chemical glycosylation (succinimidyl lysine chemistry), PEGylation of α-CT provided an excellent opportunity to compare the biophysical effects of two chemically different modifiers on the same protein system, allowing for verification of the modification-induced steric dielectric shielding proposal. For these experiments α-CT was PEGylated to different extents with PEGs of different molecular masses, recreating the experimental setup employed by Solá and Griebenow in their chemical glycosylation studies. The PEG-α-CT conjugates were then biophysically characterized to examine the effects of the PEGylation degree and PEG size on α-CT structure, dynamics, thermostability, and function. While no significant changes on α-CT tertiary structure were observed upon PEGylation, thermodynamic stability (T_m) increased with a decrease in enzymatic activity (k_{cat}) at increasing PEGylation degree, independent of PEG molecular mass [J. A. Rodríguez-Martínez, R. J. Solá, and K. Griebenow, unpublished observations]. Examination of the protein structural dynamics upon PEGylation revealed a non-linear reduction in conformational dynamics at increasing PEGylation degree independent of the PEG molecular mass. Since these results from PEGylation agree with those reported by Solá and Griebenow for glycosylation, they thus confirm the general chemical nature of the modification-induced protein desolvation proposal. Interestingly, as can be expected from such chemically different moieties, not all biophysical effects were similar. For example, while glycosylation did not alter the substrate binding affinities (K_M), increases in the PEGylation degree lead to a decrease in substrate binding affinity. Additionally, while changes in biophysical properties were altered in a linear fashion by chemical glycosylation, changes due to PEGylation behaved logarithmically. These differences can be understood if one considers that the structural composition of PEG in solution has been found to be of a compact globular form in contrast to the semi-linear nature of glycans [202, 203]. This in the context of the modification-induced protein desolvation theory would suggest that the effects of PEGylation should be more significant at lower modification degrees,

since PEG should be able to cover a larger protein surface area than glycans. While these results indicate that PEGylation could lead to increased effects at lower modification degrees when compared to glycosylation (which would be desirable for the thermodynamic stabilization of proteins), they also indicate that it is not as effective as is chemical glycosylation for the systematic modulation of protein structural dynamics. This is due to the fact that PEGylation leads to a drastic shift in protein properties upon attachment of the first two PEG chains with the effects being kept more or less constant at increasingly larger levels of modification. These studies have thus provided the first complete description of the effects of PEGylation on protein biophysical properties. Additionally, the results from these studies have provided the first complete comparative description of the effects that different chemical modifications can have on the properties of a single protein, therefore furthering the understanding of the inherent complexities involved in the tuning of protein biophysical properties.

Concluding remarks

In this review we have provided an updated account of the influence that glycosylation has on protein biophysical properties. With recent advances in glycosylation chemistry, it has now become possible to probe the mechanisms by which glycosylation alters certain protein biophysical properties through chemical glycosylation [30, 56, 57]. This has revealed the general chemical nature of these effects by glycans, affording control over such fundamental biophysical properties as protein structural dynamics, thermodynamic stability, and enzymatic function. In turn, by systematically perturbing protein structural dynamics through chemical glycosylation, it has now become possible to confirm such fundamental concepts as its influence on modulating enzyme catalysis and protein long-term stability. The mechanistic realization that these effects are caused by modulation of the protein's internal non-covalent forces due to shielding of the protein surface from the bulk solvent has now opened the way to creation of novel chemical entities for the targeted control of protein function and stability within biochemical and biomedical applications. Yet several issues concerning glycoprotein biophysics remain unresolved. For example, the mechanistic basis of glycan-promoted protein folding remains unknown. Additionally, while the biophysical effects of glycosylation amount and glycan size are now understood, the structural diversity presented by glycans is such that several structural parameters (e.g., glycan charge state) remain unexplored. Addressing these issues will require further advances in the chemical, biophysical, and computational methods currently employed for the preparation and analysis of glycoprotein structures.

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1 Mann, M. and Jensen, O. N. (2003) Proteomic analysis of post-translational modifications. Nat. Biotechnol.. 21, 255 – 261.
2 Walsh, C. T., Garneau-Tsodikova, S. and Gatto, G. J. (2005) Protein posttranslational modifications: The chemistry of proteome diversifications. Ang. Chem. Int. Ed. Engl. 44, 7342 – 7372.
3 Apweiler, R., Hermjakob, H. and Sharon, N. (1999) On the frequency of protein glycosylation, as deduced from analysis of the SWISS-PROT database. Biochim. Biophys. Acta 1473, 4 – 8.
4 Rademacher, T. W., Parekh, R. B. and Dwek, R. A. (1988) Glycobiology. Annu. Rev. Biochem. 57, 785 – 838.
5 Varki, A. (1993) Biological roles of oligosaccharides: all of the theories are correct. Glycobiology 3, 97 – 130.
6 Drickamer, K. and Taylor, M. E. (1998) Evolving views of protein glycosylation. Trends Biochem. Sci. 23, 321 – 324.
7 Reuter, G. and Gabius, H. J. (1999) Eukaryotic glycosylation: whim of nature or multipurpose tool? Cell. Mol. Life Sci. 55, 368 – 422.
8 Lowe, J. B. and Marth, J. D. (2003) A genetic approach to Mammalian glycan function. Annu. Rev. Biochem. 72, 643 – 691.
9 Weerapana, E. and Imperiali, B. (2006) Asparagine-linked protein glycosylation: from eukaryotic to prokaryotic systems. Glycobiology 16, 91R-101R.
10 Lehle, L., Strahl, S. and Tanner, W. (2006) Protein glycosylation, conserved from yeast to man: a model organism helps elucidate congenital human diseases. Angew. Chem. Int. Ed. Engl.. 45, 6802 – 6818.
11 Hounsell, E. F. and Davies, M. J. (1993) Role of protein glycosylation in immune regulation. Ann. Rheum. Dis. 52, S22 – S29.
12 Schachter, H. (2001) The clinical relevance of glycobiology. J. Clin. Invest. 108, 1579 – 1582.
13 Mitra, N., Sinha, S., Ramya, T. N. and Surolia, A. (2006) N-linked oligosaccharides as outfitters for glycoprotein folding, form and function. Trends Biochem. Sci. 31, 156 – 163.
14 Ono, M. and Hakomori, S. (2004) Glycosylation defining cancer cell motility and invasiveness. Glycoconj. J. 20, 71 – 78.
15 Sperandio, M. (2006) Selectins and glycosyltransferases in leukocyte rolling in vivo. FEBS J. 273, 4377 – 4389.
16 Lis, H. and Sharon, N. (1993) Protein glycosylation: structural and functional aspects. Eur. J. Biochem. 218, 1 – 27.
17 Paulson, J. C. (1989) Glycoproteins: what are the sugar chains for? Trends Biochem. Sci. 14, 272 – 276.
18 Borman, S. (2006) Glycosylation engineering – controlling personalities tame wild sugars on proteins and natural products. Chem. Eng. News. 84, 13 – 22.
19 Sears, P. and Wong, C. H. (1998) Enzyme action in glycoprotein synthesis. Cell. Mol. Life Sci. 54, 223 – 252.
20 Rudd, P. M. and Dwek, R. A. (1997) Glycosylation: heterogeneity and the 3D structure of proteins. Crit. Rev. Biochem. Mol. Biol. 32, 1 –100.
21 Imperiali, B. (1997) Protein glycosylation: the clash of the titans. Acc. Chem. Res. 30, 452 – 459.

22 Davis, B. G., Lloyd, R. C. and Jones, J. B. (1998) Controlled site-selective glycosylation of proteins by a combined site-directed mutagenesis and chemical modification approach. J. Org. Chem. 63, 9614 – 9615.
23 Hang, H. C. and Bertozzi, C. R. (2001) Chemoselective approaches to glycoprotein assembly. Acc. Chem. Res. 34, 727 – 736.
24 Davis, B. G. (2002) Synthesis of glycoproteins. Chem. Rev. 102, 579 – 601.
25 Grogan, M. J., Pratt, M. R., Marcaurelle, L. A. and Bertozzi, C. R. (2002) Homogeneous glycopeptides and glycoproteins for biological investigation. Annu. Rev. Biochem. 71, 593 – 634.
26 Davis, B. G. (2004) Biochemistry – Mimicking posttranslational modifications of proteins. Science 303, 480 – 482.
27 Wong, C. H. (2005) Protein glycosylation: new challenges and opportunities. J. Org. Chem. 70, 4219 – 4225.
28 Sinclair, A. M. and Elliott, S. (2005) Glycoengineering: the effect of glycosylation on the properties of therapeutic proteins. J. Pharm. Sci. 94, 1626 – 1635.
29 Pratt, M. R. and Bertozzi, C. R. (2005) Synthetic glycopeptides and glycoproteins as tools for biology. Chem. Soc. Rev. 34, 58 – 68.
30 Solá, R. J. and Griebenow, K. (2006) Chemical glycosylation: new insights on the interrelation between protein structural mobility, thermodynamic stability, and catalysis. FEBS Lett. 580, 1685 – 1690.
31 Dwek, R. A. (1995) Glycobiology: more functions for oligosaccharides. Science 269, 1234 – 1235.
32 Imperiali, B. and O'Connor, S. E. (1999) Effect of N-linked glycosylation on glycopeptide and glycoprotein structure. Curr. Opin. Chem. Biol. 3, 643 – 649.
33 Hammarsten, O. (1888) Ueber das Mucin der Submaxillardruese Darstellung. Zusammensetzung u. Eigenschaften des Submaxilaris Mucin. Z. physiol. Chem. 12, 163.
34 Rimington, C. (1929) The isolation of a carbohydrate derivative from serum-proteins. Biochem. J. 23, 430 – 443.
35 Neuberger, A. (1938) Carbohydrates in protein: the carbohydrate component of crystalline egg albumin. Biochem. J. 32, 1435 – 1451.
36 Sharon, N. (1997) Albert Neuberger (1908 – 96): founder of modern glycoprotein research. Glycobiology 7, x – xiii.
37 Gottschalk, A. (1963) The basic structure of glycoproteins and problems of their chemical and physiochemical analysis. Ann. N. Y. Acad. Sci. 106, 168 – 176.
38 Puett, D. (1973) Conformational studies on a glycosylated bovine pancreatic ribonuclease. J. Biol. Chem. 248, 3566 – 3572.
39 Montreuil, J. (1984) Spatial conformation of glycans and glycoproteins. Biol. Cell. 51, 115 – 131.
40 Gerken, T. A. (1986) The solution structure of mucous glycoproteins: proton NMR studies of native and modified ovine submaxillary mucin. Arch. Biochem. Biophys. 247, 239 – 253.
41 Gerken, T. A. and Jentoft, N. (1987) Structure and dynamics of porcine submaxillary mucin as determined by natural abundance carbon-13 NMR spectroscopy. Biochemistry 26, 4689 – 4699.
42 Shogren, R., Gerken, T. A. and Jentoft, N. (1989) Role of glycosylation on the conformation and chain dimensions of O-linked glycoproteins: light-scattering studies of ovine submaxillary mucin. Biochemistry 28, 5525 – 5536.
43 Gerken, T. A., Butenhof, K. J. and Shogren, R. (1989) Effects of glycosylation on the conformation and dynamics of O-linked glycoproteins: carbon-13 NMR studies of ovine submaxillary mucin. Biochemistry 28, 5536 – 5543.
44 Perez-Vilar, J. and Hill, R. L. (1999) The structure and assembly of secreted mucins. J. Biol. Chem. 274, 31751 – 31754.
45 Joao, H. C., Scragg, I. G. and Dwek, R. A. (1992) Effects of glycosylation on protein conformation and amide proton exchange rates in RNase B. FEBS Lett. 307, 343 – 346.
46 Joao, H. C. and Dwek, R. A. (1993) Effects of glycosylation on protein structure and dynamics in ribonuclease B and some of its individual glycoforms. Eur. J. Biochem. 218, 239 – 244.
47 Rudd, P. M., Joao, H. C., Coghill, E., Fiten, P., Saunders, M. R., Opdenakker, G. and Dwek, R. A. (1994) Glycoforms modify the dynamic stability and functional activity of an enzyme. Biochemistry 33, 17 – 22.
48 Raines, R. T. (1998) Ribonuclease A. Chem. Rev. 98, 1045 – 1066.
49 Rudd, P. M., Woods, R. J., Wormald, M. R., Opdenakker, G., Downing, A. K., Campbell, I. D. and Dwek, R. A. (1995) The effects of variable glycosylation on the functional activities of ribonuclease, plasminogen and tissue plasminogen activator. Biochim. Biophys. Acta 1248, 1 – 10.
50 Wyss, D. F., Choi, J. S., Li, J., Knoppers, M. H., Willis, K. J., Arulanandam, A. R., Smolyar, A., Reinherz, E. L. and Wagner, G. (1995) Conformation and function of the N-linked glycan in the adhesion domain of human CD2. Science 269, 1273 – 1278.
51 De Beer, T., Van Zuylen, C. W., Leeflang, B. R., Hard, K., Boelens, R., Kaptein, R., Kamerling, J. P. and Vliegenthart, J. F. (1996) NMR studies of the free alpha subunit of human chorionic gonadotropin. Structural influences of N-glycosylation and the beta subunit on the conformation of the alpha subunit. Eur. J. Biochem. 241, 229 – 242.
52 Mer, G., Hietter, H. and Lefevre, J. F. (1996) Stabilization of proteins by glycosylation examined by NMR analysis of a fucosylated proteinase inhibitor. Nat. Struct. Biol. 3, 45 – 53.
53 O'Connor, S. E. and Imperiali, B. (1996) Modulation of protein structure and function by asparagine-linked glycosylation. Chem. Biol. 3, 803 – 812.
54 Wyss, D. F. and Wagner, G. (1996) The structural role of sugars in glycoproteins. Curr. Opin. Biotechnol. 7, 409 – 416.
55 Wormald, M. R. and Dwek, R. A. (1999) Glycoproteins: glycan presentation and protein-fold stability. Structure Fold Des. 7, R155 – R160.
56 Solá, R. J., Al-Azzam, W. and Griebenow, K. (2006) Engineering of protein thermodynamic, kinetic, and colloidal stability: chemical glycosylation with monofunctionally activated glycans. Biotechnol. Bioeng. 94, 1072 – 1079.
57 Solá, R. J. and Griebenow, K. (2006) Influence of modulated structural dynamics on the kinetics of alpha-chymotrypsin catalysis. Insights through chemical glycosylation, molecular dynamics, and domain motion analysis. FEBS J. 273, 5303 – 5319.
58 Hermanson, G. T. (1996) Bioconjugate Techniques. Academic Press, New York.
59 Vegarud, G. and Christnsen, T. B. (1975) Glycosylation of proteins: a new method of enzyme stabilization. Biotechnol. Bioeng. 17, 1391 – 1397.
60 Wang, C., Eufemi, M., Turano, C. and Giartosio, A. (1996) Influence of the carbohydrate moiety on the stability of glycoproteins. Biochemistry 35, 7299 – 7307.
61 DeKoster, G. T. and Robertson, A. D. (1997) Thermodynamics of unfolding for Kazal-type serine protease inhibitors: entropic stabilization of ovomucoid first domain by glycosylation. Biochemistry 36, 2323 – 2331.
62 Kwon, K. S. and Yu, M. H. (1997) Effect of glycosylation on the stability of alpha1-antitrypsin toward urea denaturation and thermal deactivation. Biochim. Biophys. Acta 1335, 265 – 272.
63 Leong, S. S. and Middelberg, A. P. (2006) The refolding of different alpha-fetoprotein variants. Protein Sci. 15, 2040 – 2050.
64 Kimura, N., Uchida, M., Nishimura, S. and Yamaguchi, H. (1998) Promotion of polypeptide folding by interactions with Asn-Glycans. J. Biochem. (Tokyo). 124, 857 – 862.
65 Yamaguchi, H. (1999) Direct promotion of polypeptide folding by Asn-glycans. Seikagaku 71, 201 – 205.
66 Yamaguchi, H. (2002) Chaperone-like functions of N-glycans in the formation and stabilization of protein conformation. Trends Glycosci. Glycotechnol. 14, 139 – 151.

67 Mitra, N., Sharon, N. and Surolia, A. (2003) Role of N-linked glycan in the unfolding pathway of Erythrina corallodendron lectin. Biochemistry.42, 12208 – 12216.
68 Kulkarni, K. A., Srivastava, A., Mitra, N., Sharon, N., Surolia, A., Vijayan, M. and Suguna, K. (2004) Effect of glycosylation on the structure of Erythrina corallodendron lectin. Proteins 56, 821 – 827.
69 Sinha, S. and Surolia, A. (2007) Attributes of glycosylation in the establishment of the unfolding pathway of soybean agglutinin. Biophys. J. 92, 208 – 216.
70 Hackenberger, C. P., Friel, C. T., Radford, S. E. and Imperiali, B. (2005) Semisynthesis of a glycosylated Im7 analogue for protein folding studies. J. Am. Chem. Soc. 127, 12882 – 12889.
71 Broersen, K., Voragen, A. G., Hamer, R. J. and De Jongh, H. H. (2004) Glycoforms of beta-lactoglobulin with improved thermostability and preserved structural packing. Biotechnol. Bioeng. 86, 78 – 87.
72 Van Teeffelen, A. M. M., Broersen, K. and De Jongh, H. H. J. (2005) Glucosylation of beta-lactoglobulin lowers the heat capacity change of unfolding; a unique way to affect protein thermodynamics. Protein Sci. 14, 2187 – 2194.
73 Grinnell, B. W., Walls, J. D. and Gerlitz, B. (1991) Glycosylation of human protein C affects its secretion, processing, functional activities, and activation by thrombin. J. Biol. Chem. 266, 9778 – 9785.
74 Parekh, R. B., Dwek, R. A., Rudd, P. M., Thomas, J. R., Rademacher, T. W., Warren, T., Wun, T. C., Hebert, B., Reitz, B., Palmier, M. et al. (1989) N-glycosylation and in vitro enzymatic activity of human recombinant tissue plasminogen activator expressed in Chinese hamster ovary cells and a murine cell line. Biochemistry 28, 7670 – 7679.
75 Rademacher, T. W. and Dwek, R. A. (1989) The role of oligosaccharides in modifying protein function. Ciba Found Symp. 145, 241 – 255; discussion 255 – 256.
76 Kohen, A., Jonsson, T. and Klinman, J. P. (1997) Effects of protein glycosylation on catalysis: changes in hydrogen tunneling and enthalpy of activation in the glucose oxidase reaction. Biochemistry 36, 2603 – 2611.
77 Agarwal, P. K. (2006) Enzymes: an integrated view of structure, dynamics and function. Microb. Cell Fact. 5, 2.
78 Damborsky, J. (1998) Quantitative structure-function and structure-stability relationships of purposely modified proteins. Protein Eng. 11, 21 – 30.
79 Privalov, P. L. and Tsalkova, T. N. (1979) Micro- and macrostabilities of globular proteins. Nature 280, 693 – 696.
80 Damjanovich, S., Somogyi, B. and Welch, G. R. (1983) Protein fluctuation and enzyme activity. J. Theor. Biol. 105, 25 – 33.
81 Berendsen, H. J. and Hayward, S. (2000) Collective protein dynamics in relation to function. Curr. Opin. Struct. Biol. 10, 165 – 169.
82 Haouz, A., Glandieres, J. M. and Alpert, B. (2001) Involvement of protein dynamics in enzyme stability. The case of glucose oxidase. FEBS Lett. 506, 216 – 220.
83 Tsai, A. M., Udovic, T. J. and Neumann, D. A. (2001) The inverse relationship between protein dynamics and thermal stability. Biophys. J. 81, 2339 – 2343.
84 Livesay, D. R., Dallakyan, S., Wood, G. G. and Jacobs, D. J. (2004) A flexible approach for understanding protein stability. FEBS Lett. 576, 468 – 476.
85 Scrutton, N. S., Basran, J. and Sutcliffe, M. J. (1999) New insights into enzyme catalysis. Ground state tunnelling driven by protein dynamics. Eur. J. Biochem. 264, 666 – 671.
86 Wand, A. J. (2001) Dynamic activation of protein function: a view emerging from NMR spectroscopy. Nat. Struct. Biol. 8, 926 – 931.
87 Antoniou, D., Caratzoulas, S., Kalyanaraman, C., Mincer, J. S. and Schwartz, S. D. (2002) Barrier passage and protein dynamics in enzymatically catalyzed reactions. Eur. J. Biochem. 269, 3103 – 3112.
88 Knapp, M. J. and Klinman, J. P. (2002) Environmentally coupled hydrogen tunneling. Linking catalysis to dynamics. Eur. J. Biochem. 269, 3113 – 3121.
89 Hammes-Schiffer, S. (2002) Impact of enzyme motion on activity. Biochemistry 41, 13335 – 13343.
90 Hammes-Schiffer, S. and Benkovic, S. J. (2006) Relating protein motion to catalysis. Annu. Rev. Biochem. 75, 519 – 541.
91 Benkovic, S. J. and Hammes-Schiffer, S. (2003) A perspective on enzyme catalysis. Science 301, 1196 – 1202.
92 Benkovic, S. J. and Hammes-Schiffer, S. (2006) Biochemistry. Enzyme motions inside and out. Science 312, 208 – 209.
93 Ball, P. (2006) Chemistry: what chemists want to know. Nature 442, 500 – 502.
94 Zavodszky, P., Kardos, J., Svingor and Petsko, G. A. (1998) Adjustment of conformational flexibility is a key event in the thermal adaptation of proteins. Proc. Natl. Acad. Sci. USA 95, 7406 – 7411.
95 Volkman, B. F., Lipson, D., Wemmer, D. E. and Kern, D. (2001) Two-state allosteric behavior in a single-domain signaling protein. Science 291, 2429 – 2433.
96 Case, A. and Stein, R. L. (2003) Mechanistic origins of the substrate selectivity of serine proteases. Biochemistry 42, 3335 – 3348.
97 Hedstrom, L. (2002) Serine protease mechanism and specificity. Chem. Rev. 102, 4501 – 4524.
98 Hengge, A. C. and Stein, R. L. (2004) Role of protein conformational mobility in enzyme catalysis: acylation of alpha-chymotrypsin by specific peptide substrates. Biochemistry 43, 742 – 747.
99 Lumry, R. (1974) Conformational mechanisms for free energy transduction in protein systems: old ideas and new facts. Ann. N. Y. Acad. Sci. 227, 46 – 73.
100 Somogyi, B., Welch, G. R. and Damjanovich, S. (1984) The dynamic basis of energy transduction in enzymes. Biochim. Biophys. Acta 768, 81 – 112.
101 Kurzynski, M. (1997) Protein machine model of enzymatic reactions gated by enzyme internal dynamics. Biophys.Chem. 65, 1 – 28.
102 Garcia-Viloca, M., Gao, J., Karplus, M. and Truhlar, D. G. (2004) How enzymes work: analysis by modern rate theory and computer simulations. Science 303, 186 – 195.
103 Astumian, R. D., Chock, P. B., Tsong, T. Y. and Westerhoff, H. V. (1989) Effects of oscillations and energy-driven fluctuations on the dynamics of enzyme catalysis and free-energy transduction. Phys. Rev. A. 39, 6416 – 6435.
104 Eisenmesser, E. Z., Bosco, D. A., Akke, M. and Kern, D. (2002) Enzyme dynamics during catalysis. Science 295, 1520 – 1523.
105 Agarwal, P. K., Billeter, S. R., Rajagopalan, P. T., Benkovic, S. J. and Hammes-Schiffer, S. (2002) Network of coupled promoting motions in enzyme catalysis. Proc. Natl. Acad. Sci. US. 99, 2794 – 2799.
106 Yang, L. W. and Bahar, I. (2005) Coupling between catalytic site and collective dynamics: a requirement for mechanochemical activity of enzymes. Structure 13, 893 – 904.
107 Eisenmesser, E. Z., Millet, O., Labeikovsky, W., Korzhnev, D. M., Wolf-Watz, M., Bosco, D. A., Skalicky, J. J., Kay, L. E. and Kern, D. (2005) Intrinsic dynamics of an enzyme underlies catalysis. Nature 438, 117 – 121.
108 Hawkins, R. J. and McLeish, T. C. (2006) Coupling of global and local vibrational modes in dynamic allostery of proteins. Biophys. J. 91, 2055 – 2062.
109 Gunasekaran, K., Ma, B. and Nussinov, R. (2004) Is allostery an intrinsic property of all dynamic proteins? Proteins 57, 433 – 443.
110 Hawkins, R. J. and McLeish, T. C. (2004) Coarse-grained model of entropic allostery. Phys. Rev. Lett. 93, 098104.
111 Wand, A. J. (2001) On the dynamic origins of allosteric activation. Science 293, 1395.
112 Cooper, A. and Dryden, D. T. (1984) Allostery without conformational change. A plausible model. Eur. Biophys. J. 11, 103 – 109.

113 Popovych, N., Sun, S., Ebright, R. H. and Kalodimos, C. G. (2006) Dynamically driven protein allostery. Nat. Struct. Mol. Biol. 13, 831 – 838.
114 Perutz, M. F. (1989) Mechanisms of cooperativity and allosteric regulation in proteins. Q. Rev. Biophys. 22, 139 – 237.
115 Pace, C. N. (1990) Measuring and increasing protein stability. Trends Biotechnol. 8, 93 – 98.
116 Krieger, E., Darden, T., Nabuurs, S. B., Finkelstein, A. and Vriend, G. (2004) Making optimal use of empirical energy functions: force-field parameterization in crystal space. Proteins 57, 678 – 683.
117 Wang, J. M., Cieplak, P. and Kollman, P. A. (2000) How well does a restrained electrostatic potential (RESP) model perform in calculating conformational energies of organic and biological molecules? J. Comput. Chem. 21, 1049 – 1074.
118 Woods, R. J., Dwek, R. A., Edge, C. J. and Fraserreid, B. (1995) Molecular mechanical and molecular dynamical simulations of glycoproteins and oligosaccharides. 1. Glycam-93 parameter development. J. Phys. Chem. 99, 3832 – 3846.
119 Kundu, S., Melton, J. S., Sorensen, D. C. and Phillips, G. N., Jr. (2002) Dynamics of proteins in crystals: comparison of experiment with simple models. Biophys. J. 83, 723 – 732.
120 Affleck, R., Haynes, C. A. and Clark, D. S. (1992) Solvent dielectric effects on protein dynamics. Proc. Natl. Acad. Sci. USA 89, 5167 – 5170.
121 Simonson, T. (2003) Elec, P. W., Frauenfelder, H., McMahon, B. H. and Parak, F. G. (2002) Slaving: solvent fluctuations dominate protein dynamics and functions. Proc. Natl. Acad. Sci. USA 99, 16047 – 16051.
122 Strickler, S. S., Gribenko, A. V., Gribenko, A. V., Keiffer, T. R., Tomlinson, J., Reihle, T., Loladze, V. V. and Makhatadze, G. I. (2006) Protein stability and surface electrostatics: a charged relationship. Biochemistry 45, 2761 – 2766.
123 Fenimore, P. W., Frauenfelder, H., McMahon, B. H., and Parak, F. G. (2002) Slaving: solvent fluctuations dominate protein dynamics and functions. Proc. Natl. Acad. Sci. USA 99, 16047 – 16051.
124 Fenimore, P. W., Frauenfelder, H., McMahon, B. H. and Young, R. D. (2004) Bulk-solvent and hydration-shell fluctuations, similar to alpha- and beta-fluctuations in glasses, control protein motions and functions. Proc. Natl. Acad. Sci. USA 101, 14408 – 14413.
125 Havranek, J. J. and Harbury, P. B. (1999) Tanford-Kirkwood electrostatics for protein modeling. Proc. Natl. Acad. Sci. USA 96, 11145 – 11150.
126 Rubinstein, A. and Sherman, S. (2004) Influence of the solvent structure on the electrostatic interactions in proteins. Biophys. J. 87, 1544 – 1557.
127 Zaks, A. and Klibanov, A. M. (1988) The effect of water on enzyme action in organic media. J. Biol. Chem. 263, 8017 – 8021.
128 Rupley, J. A. and Careri, G. (1991) Protein hydration and function. Adv. Protein Chem. 41, 37 – 172.
129 Klibanov, A. M. (1997) Why are enzymes less active in organic solvents than in water? Trends Biotechnol. 15, 97 – 101.
130 Partridge, J., Dennison, P. R., Moore, B. D. and Halling, P. J. (1998) Activity and mobility of subtilisin in low water organic media: hydration is more important than solvent dielectric. Biochim. Biophys. Acta 1386, 79 – 89.
131 Griebenow, K., Vidal, M., Baez, C., Santos, A. M. and Barletta, G. (2001) Nativelike enzyme properties are important for optimum activity in neat organic solvents. J. Am. Chem. Soc. 123, 5380 – 5381.
132 Yang, L., Dordick, J. S. and Garde, S. (2004) Hydration of enzyme in nonaqueous media is consistent with solvent dependence of its activity. Biophys. J. 87, 812 – 821.
133 Clark, D. S. (2004) Characteristics of nearly dry enzymes in organic solvents: implications for biocatalysis in the absence of water. Philos. Trans. R. Soc. Lond. B Biol. Sci. 359, 1299 – 1307; discussion 1307, 1323 – 1328.
134 Castillo, B., Bansal, V., Ganesan, A., Halling, P., Secundo, F., Ferrer, A., Griebenow, K. and Barletta, G. (2006) On the activity loss of hydrolases in organic solvents: II. a mechanistic study of subtilisin Carlsberg. BMC Biotechnol. 6, 51.
135 Luscher, M., Ruegg, M. and Schindler, P. (1978) Thermodynamic studies of the interaction of alpha-chymotrypsin with water. II. Statistical analyses of the enthalpy-entropy compensation effect. Biochim. Biophys. Acta 536, 27 – 37.
136 Dunitz, J. D. (1995) Win some, lose some: enthalpy-entropy compensation in weak intermolecular interactions. Chem. Biol. 2, 709 – 712.
137 Lumry, R. (2003) Uses of enthalpy-entropy compensation in protein research. Biophys. Chem. 105, 545 – 557.
138 Pal, S. K., Peon, J. and Zewail, A. H. (2002) Ultrafast surface hydration dynamics and expression of protein functionality: alpha-Chymotrypsin. Proc. Natl. Acad. Sci. USA 99, 15297 – 15302.
139 Bruice, T. C. and Benkovic, S. J. (2000) Chemical basis for enzyme catalysis. Biochemistry 39, 6267 – 6274.
140 Frauenfelder, H. and McMahon, B. (1998) Dynamics and function of proteins: the search for general concepts. Proc. Natl. Acad. Sci. USA 95, 4795 – 4797.
141 Blow, D. (2000) So do we understand how enzymes work? Structure 8, R77 – R81.
142 Careri, G., Fasella, P. and Gratton, E. (1979) Enzyme dynamics: the statistical physics approach. Annu. Rev. Biophys. Bioeng. 8, 69 – 97.
143 Careri, G., Fasella, P. and Gratton, E. (1975) Statistical time events in enzymes: a physical assessment. CRC Crit. Rev. Biochem. 3, 141 – 164.
144 Peticolas, W. L. (1979) Low frequency vibrations and the dynamics of proteins and polypeptides. Methods Enzymol. 61, 425 – 458.
145 Rawlings, N. D., Morton, F. R. and Barrett, A. J. (2006) MEROPS: the peptidase database. Nucleic Acids Res. 34, D270 – D272.
146 Gerstein, M., Lesk, A. M. and Chothia, C. (1994) Structural mechanisms for domain movements in proteins. Biochemistry 33, 6739 – 6749.
147 Dufton, M. J. (1990) Could domain movements be involved in the mechanism of trypsin-like serine proteases? FEBS Lett. 271, 9 – 13.
148 Chen, S. C. and Bahar, I. (2004) Mining frequent patterns in protein structures: a study of protease families. Bioinformatics 20 Suppl. 1, I177 – I185.
149 Ma, W., Tang, C. and Lai, L. (2005) Specificity of trypsin and chymotrypsin: loop-motion-controlled dynamic correlation as a determinant. Biophys. J. 89, 1183 – 1193.
150 Yang, L. W., Liu, X., Jursa, C. J., Holliman, M., Rader, A. J., Karimi, H. A. and Bahar, I. (2005) iGNM: a database of protein functional motions based on Gaussian Network Model. Bioinformatics 21, 2978 – 2987.
151 Bahar, I., Erman, B., Haliloglu, T. and Jernigan, R. L. (1997) Efficient characterization of collective motions and interresidue correlations in proteins by low-resolution simulations. Biochemistry 36, 13512 – 13523.
152 Haliloglu, T. and Bahar, I. (1999) Structure-based analysis of protein dynamics: comparison of theoretical results for hen lysozyme with X-ray diffraction and NMR relaxation data. Proteins 37, 654 – 667.
153 Bahar, I., Wallqvist, A., Covell, D. G. and Jernigan, R. L. (1998) Correlation between native-state hydrogen exchange and cooperative residue fluctuations from a simple model. Biochemistry 37, 1067 – 1075.
154 Ishida, T. (2006) Low-barrier hydrogen bond hypothesis in the catalytic triad residue of serine proteases: correlation between structural rearrangement and chemical shifts in the acylation process. Biochemistry 45, 5413 – 5420.
155 Carnevale, V., Raugei, S., Micheletti, C. and Carloni, P. (2006) Convergent dynamics in the protease enzymatic superfamily. J. Am. Chem. Soc. 128, 9766 – 9772.

156 Andersen, D. C. and Krummen, L. (2002) Recombinant protein expression for therapeutic applications. Curr. Opin. Biotechnol. 13, 117 – 123.
157 Illanes, A. (1999) Stability of biocatalysts. Electronic Journal of Biotechnology 2, 1 – 9.
158 Marshall, S. A., Lazar, G. A., Chirino, A. J. and Desjarlais, J. R. (2003) Rational design and engineering of therapeutic proteins. Drug Discov.Today 8, 212 – 221.
159 Perez, C., Castellanos, I. J., Costantino, H. R., Al-Azzam, W. and Griebenow, K. (2002) Recent trends in stabilizing protein structure upon encapsulation and release from bioerodible polymers. J. Pharm. Pharmacol. 54, 301 – 313.
160 Wang, W. (1999) Instability, stabilization, and formulation of liquid protein pharmaceuticals. Int. J. Pharm. 185, 129 – 188.
161 Wang, W. (2000) Lyophilization and development of solid protein pharmaceuticals. Int. J. Pharm. 203, 1 – 60.
162 Frokjaer, S. and Otzen, D. E. (2005) Protein drug stability: a formulation challenge. Nat. Rev. Drug Discov. 4, 298 – 306.
163 Byrn, S. R., Xu, W. and Newman, A. W. (2001) Chemical reactivity in solid-state pharmaceuticals: formulation implications. Adv. Drug Deliv. Rev. 48, 115 – 136.
164 Volkin, D. B., Sanyal, G., Burke, C. J. and Middaugh, C. R. (2002) Preformulation studies as an essential guide to formulation development and manufacture of protein pharmaceuticals. Pharm. Biotechnol. 14, 1 – 46.
165 Volkin, D. B., Mach, H. and Middaugh, C. R. (1997) Degradative covalent reactions important to protein stability. Mol. Biotechnol. 8, 105 – 122.
166 Pace, C. N. (1990) Conformational stability of globular proteins. Trends Biochem. Sci. 15, 14 – 17.
167 Valente, J. J., Payne, R. W., Manning, M. C., Wilson, W. W. and Henry, C. S. (2005) Colloidal behavior of proteins: effects of the second virial coefficient on solubility, crystallization and aggregation of proteins in aqueous solution. Curr. Pharm. Biotechnol. 6, 427 – 436.
168 Chi, E. Y., Krishnan, S., Kendrick, B. S., Chang, B. S., Carpenter, J. F. and Randolph, T. W. (2003) Roles of conformational stability and colloidal stability in the aggregation of recombinant human granulocyte colony-stimulating factor. Protein Sci. 12, 903 – 913.
169 Privalov, P. L. (1979) Stability of proteins: small globular proteins. Adv. Protein Chem. 33, 167 – 241.
170 Davis, B. G. (2003) Chemical modification of biocatalysts. Curr. Opin. Biotechnol. 14, 379 – 386.
171 Vegarud, G. and Christensen, T. B. (1975) The resistance of glycoproteins to proteolytic inactivation. Acta Chem. Scand. B 29, 887 – 888.
172 Holcenberg, J. S., Schmer, G. and Teller, D. C. (1975) Biologic and physical properties of succinylated and glycosylated Acinetobacter glutaminase-asparaginase. J. Biol. Chem. 250, 4165 – 4170.
173 Dellacherie, E., Bonneaux, F., Labrude, P. and Vigneron, C. (1983) Modification of human hemoglobin by covalent association with soluble dextran. Biochim. Biophys. Acta 749, 106 – 114.
174 Lenders, J. P. and Chricton, R. R. (1984) Thermal stabilization of amylolytic enzymes by covalent coupling to soluble polysaccharides. Biotechnol. Bioeng. 26, 1343 – 1351.
175 Srivastava, R. A. K. (1991) Studies on stabilization of amylase by covalent coupling to soluble polysaccharides. Enzyme Microbial Technol. 13, 164 – 170.
176 Baudys, M., Uchio, T., Mix, D., Wilson, D. and Kim, S. W. (1995) Physical stabilization of insulin by glycosylation. J. Pharm. Sci. 84, 28 – 33.
177 Hoiberg-Nielsen, R., Fuglsang, C. C., Arleth, L. and Westh, P. (2006) Interrelationships of glycosylation and aggregation kinetics for Peniophora lycii phytase. Biochemistry 45, 5057 – 5066.
178 Bagger, H. L., Fuglsang, C. C. and Westh, P. (2003) Preferential binding of two compatible solutes to the glycan moieties of Peniophora lycii phytase. Biochemistry 42, 10295 – 10300.
179 Bagger, H. L., Fuglsang, C. C. and Westh, P. (2006) Hydration of a glycoprotein: relative water affinity of peptide and glycan moieties. Eur. Biophys. J. 35, 367 – 371.
180 Gregoriadis, G., Fernandes, A., McCormack, B., Mital, M. and Zhang, X. (1999) Polysialic acids: potential role in therapeutic constructs. Biotechnol. Genet. Eng. Rev. 16, 203 – 215.
181 Gregoriadis, G., Fernandes, A., Mital, M. and McCormack, B. (2000) Polysialic acids: potential in improving the stability and pharmacokinetics of proteins and other therapeutics. Cell. Mol. Life Sci. 57, 1964 – 1969.
182 Gregoriadis, G., McCormack, B., Wang, Z. and Lifely, R. (1993) Polysialic acids: potential in drug delivery. FEBS Lett. 315, 271 – 276.
183 Jain, S., Hreczuk-Hirst, D. H., McCormack, B., Mital, M., Epenetos, A., Laing, P. and Gregoriadis, G. (2003) Polysialylated insulin: synthesis, characterization and biological activity in vivo. Biochim. Biophys. Acta 1622, 42 – 49.
184 Elliott, S., Egrie, J., Browne, J., Lorenzini, T., Busse, L., Rogers, N. and Ponting, I. (2004) Control of rHuEPO biological activity: the role of carbohydrate. Exp. Hematol. 32, 1146 – 1155.
185 Elliott, S., Lorenzini, T., Asher, S., Aoki, K., Brankow, D., Buck, L., Busse, L., Chang, D., Fuller, J., Grant, J. et al. (2003) Enhancement of therapeutic protein in vivo activities through glycoengineering. Nat. Biotechnol. 21, 414 – 421.
186 Duncan, R. (2003) The dawning era of polymer therapeutics. Nat. Rev. Drug Discov. 2, 347 – 360.
187 Topchieva, I. N., Efremova, N. V., Khvorov, N. V. and Magretova, N. N. (1995) Synthesis and physicochemical properties of protein conjugates with water-soluble poly(-alkylene oxides). Bioconjug. Chem. 6, 380 – 388.
188 Ton, G. N., Fine, J. P. and Kwon, G. S. (2005) Methoxypoly(-ethylene glycol)-conjugated carboxypeptidase A for solid tumor targeting. Part I: Synthesis and characterization. J. Control.Release 104, 129 – 139.
189 Ton, G. N., Weichert, J. P., Longino, M. A., Fine, J. P. and Kwon, G. S. (2005) Methoxypoly(ethylene glycol)-conjugated carboxypeptidase A for solid tumor targeting. Part II: Pharmacokinetics and biodistribution in normal and tumor-bearing rodents. J. Control. Release 104, 155 – 166.
190 Castellanos, I. J., Al-Azzam, W. and Griebenow, K. (2005) Effect of the covalent modification with poly(ethylene glycol) on alpha-chymotrypsin stability upon encapsulation in poly-(lactic-co-glycolic) microspheres. J. Pharm. Sci. 94, 327 – 340.
191 Rajan, R. S., Li, T., Aras, M., Sloey, C., Sutherland, W., Arai, H., Briddell, R., Kinstler, O., Lueras, A. M., Zhang, Y. et al. (2006) Modulation of protein aggregation by polyethylene glycol conjugation: GCSF as a case study. Protein Sci. 15, 1063 – 1075.
192 Nie, Y. J., Zhang, X., Wang, X. C. and Chen, J. H. (2006) Preparation and stability of N-terminal mono-PEGylated recombinant human endostatin. Bioconjug. Chem. 17, 995 – 999.
193 Abuchowski, A., McCoy, J. R., Palczuk, N. C., van Es, T. and Davis, F. F. (1977) Effect of covalent attachment of polyethylene glycol on immunogenicity and circulating life of bovine liver catalase. J. Biol. Chem. 252, 3582 – 3586.
194 Abuchowski, A., van Es, T., Palczuk, N. C. and Davis, F. F. (1977) Alteration of immunological properties of bovine serum albumin by covalent attachment of polyethylene glycol. J. Biol. Chem. 252, 3578 – 3581.
195 Harris, J. M. and Chess, R. B. (2003) Effect of pegylation on pharmaceuticals. Nat. Rev. Drug Discov. 2, 214 – 221.
196 Harris, J. M., Martin, N. E. and Modi, M. (2001) Pegylation – A novel process for modifying pharmacokinetics. Clin. Pharmacokinet. 40, 539 – 551.
197 Veronese, F. M. and Pasut, G. (2005) PEGylation, successful approach to drug delivery. Drug Discov.Today 10, 1451 – 1458.
198 Gilmore, J. M., Scheck, R. A., Esser-Kahn, A. P., Joshi, N. S. and Francis, M. B. (2006) N-terminal protein modification

through a biomimetic transamination reaction. Angew. Chem. Int. Ed Engl.. 45, 5307 – 5311.
199 Shaunak, S., Godwin, A., Choi, J. W., Balan, S., Pedone, E., Vijayarangam, D., Heidelberger, S., Teo, I., Zloh, M. and Brocchini, S. (2006) Site-specific PEGylation of native disulfide bonds in therapeutic proteins. Nat. Chem. Biol. 2, 312 – 313.
200 Roberts, M. J., Bentley, M. D. and Harris, J. M. (2002) Chemistry for peptide and protein PEGylation. Adv. Drug Deliv. Rev. 54, 459 – 476.
201 Greenwald, R. B. (2001) PEG drugs: an overview. J. Control. Release 74, 159 – 171.
202 Manjula, B. N., Tsai, S., Upadhya, R., Perumalsamy, K., Smith, P. K., Malavalli, A., Vandegriff, K., Winslow, R. M., Intaglietta, M., Prabhakaran, M. et al. (2003) Site-specific PEGylation of hemoglobin at cys-93(beta): Correlation between the colligative properties of the PEGylated protein and the length of the conjugated PEG chain. Bioconjug. Chem. 14, 464 – 472.
203 Hu, T., Prabhakaran, M., Acharya, S. A. and Manjula, B. N. (2005) Influence of the chemistry of conjugation of poly(-ethylene glycol) to Hb on the oxygen-binding and solution properties of the PEG-Hb conjugate. Biochem. J. 392, 555 – 564.